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Biochemical Determinants of Microbial Diseases

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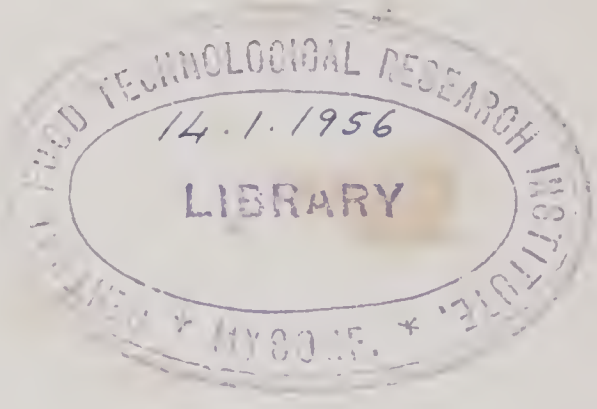

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Preface

It is common practice to refer to the diseases caused by the various groups of microorganisms as *infectious* diseases. This expression naturally puts emphasis on the propensity of microbial agents to spread from one individual to another. The event of infection, however, constitutes merely one phase of the problem of microbial diseases, for only in a very small percentage of infected individuals does the presence of microbial agents result in symptoms or pathological lesions.

The sciences of immunology, epidemiology, physiology, psychology, even of sociology, can all throw light on the influences which determine whether infection is abortive, self-limited or evolves into overt disease, but the problem will be considered here from a more limited point of view. The essays which follow deal exclusively with the biochemical factors that affect the ability of microbial agents to proliferate *in vivo* and to cause metabolic disturbances and alterations of tissues. These essays were presented in Boston on the occasion of the award of the Warren Triennal Prize of the Massachusetts General Hospital in November 1953. They constituted also part of the subject material of a series of lectures delivered in Berkeley and Los Angeles during my tenure of the Hitchcock Professorship at the University of California in the Spring of 1954.

To the Trustees of the Massachusetts General Hospital, and to the Board of Regents of the University of California, I wish to express my gratitude for having given me the opportunity to present in a friendly atmosphere these gropings toward a biochemical explanation of host-parasite relationships.

R.J.D.

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CHAPTER 1

Infection into disease

Ten years ago, I delivered under the auspices of The Lowell Institute in Boston a series of lectures entitled "The Bacterial Cell: In Its Relation to Problems of Virulence, Immunity and Chemotherapy" (Dubos, 1945). In the Lowell lectures, I dealt with the components, products, and properties of microorganisms which endow them with potential pathogenicity and immunogenic power, and which affect their susceptibility to immunity mechanisms and to chemotherapeutic agents. Little mention will be made of these problems in the present book. Instead of the infectious agent, I shall emphasize the properties of the infected host which determine the course and outcome of the infectious process. The study of the tissue factors which are responsible for arresting the progress of infection, or for allowing it to evolve into overt disease, is still in the most primitive state. Nevertheless, as it is today the center of my scientific interests, a discussion of it may serve better than a more classical, but less personal, topic, to express my gratitude for the invitation to deliver the first series of the Warren Triennal Lectures.

Before undertaking a detailed discussion of the various factors which determine the course of infectious processes, I shall attempt to outline the reasons which have oriented my interest toward this problem. This will be made easier by considering first an example taken from recent history. A group of young Danish physicians who were political prisoners in German concentration camps through part of the Second World War have written an engrossing account of the health problems which affected their

own group and other internees during captivity (Helweg *et al.*, 1953). Among infectious diseases, it was not the exotic, unusual epidemics like typhus, cholera, or even bacillary dysentery which proved the most troublesome in their experience, but rather ordinary skin ailments, colds, bronchopneumonias, staphylococcus infections, pulmonary tuberculosis — in other words, the type of diseases, minor or severe, caused by microorganisms *endemic* in the normal European communities. Increase in contact infections could hardly account for the aggravation of these endemic diseases. Far more important certainly was the loss of natural resistance caused by malnutrition and other forms of physiological misery. It was remarkable indeed that most of the internees overcame their microbial maladies shortly after their return to a normal environment and often without the help of specific therapy. Even in the case of tuberculosis, rapid recovery was the rule, though no antimicrobial agent was then available for its treatment.

This dependence of susceptibility to infection upon the physiological status of the host is of course a familiar observation. However commonplace, it focuses attention upon an aspect of infectious disease which is much neglected. Most human beings, indeed probably all living things, carry throughout life a variety of microbial agents potentially pathogenic for them. Under most conditions, these pathogens do not manifest their presence by either symptoms or lesions; only when something happens which upsets the equilibrium between host and parasite does infection evolve into disease. In other words, infection is in many cases the normal state; it is only disease which is abnormal. If space permitted, I could illustrate by many specific examples the relevance of these considerations to the problem of infection in man. It is certain that much of the burden of disease in our communities is caused by pathogenic agents which are maintained normally in "silent" infections and manifest their presence only under the stimulus of other factors of the environment. The happenings in the concentration camps are merely a magni-

fied manifestation of a state of affairs observed daily in medical practice.

Needless to say, there is an extensive literature dealing with the phenomenon of silent infection, often referred to also as carrier state, subclinical infection, or perhaps preferably latent infection (reviewed in Meyer, 1936; Andrewes, 1939, 1950, 1952; Koprowski, 1952; Smith, 1952). But latency, like resistance to, and recovery from infection, has been studied almost exclusively by immunological techniques. Humoral antibodies and cellular immunity are the forces which are most commonly invoked to define the state of equilibrium or of conflict between parasite and host. Yet the problem of the host-parasite relationship needs to be analyzed from many other points of view. Let us consider a few examples, taken almost at random from among the many where forces other than immunological ones determine the outcome of infection.

As is well known, the agent responsible for mammary carcinoma among breeding female mice has all the characteristics of a virus (Bittner, 1947). The Bittner virus can be present throughout the tissues of mice and yet cause no sign of disease and in particular no tumors until lactation begins. Male mice never develop cancer even if they carry or have ingested the virus, nor do female mice unless under the stimulus of continuous reproductive activity. Thus, the hormonal and other stimuli which accompany reproduction and lactation are necessary and sufficient to induce the virus to manifest its potential pathogenicity. In other words, these physiological stimuli are the factors which convert infection into neoplastic disease.

There exists a large body of clinical observations in man and of experiments in animals demonstrating beyond doubt that various specific hormones can modify the course of infection. Of particular interest is the fact that cortisone treatment can cause animals to develop a fatal disease owing to the multiplication of microorganisms which they normally carry in their tissues in the form of an inapparent infection. In experiments with white rats,

extensive necrotic lesions associated with the presence of *Corynebacterium pseudotuberculosis murium* were commonly found within a few weeks following the initiation of treatment with large amounts of the hormone. The corynebacteria were apparently present in the tissues of the normal animals, but in such small numbers that they could not be detected by either bacteriological or histological techniques until the physiological disturbance caused by the hormone had allowed their multiplication (Le Maistre and Tompsett, 1952). Pseudotuberculous lesions caused by corynebacteria have also been reported to occur spontaneously in albino rats maintained on deficient diets, and to regress when the deficiency was corrected by treatment with a crude ill-defined preparation designated as vitamin H (Gundel, Gyorgy, and Pagel, 1931). In very recent experiments with exactly defined diets it has been shown that pantothenic acid deficiency can evoke latent corynebacteria infections, and also render normal animals susceptible to experimental infection with these microorganisms (Zucker and Zucker, 1954; Seronde, 1954). Pseudotuberculosis caused by corynebacteria has also been observed after irradiation of mice (Schechmeister and Adler, 1953).

The number of latent virus infections which have been discovered in experimental animals and in plants is so large and their biological characteristics have been so minutely described that all aspects of the problem of latency in infectious diseases could be illustrated with examples taken from this field. Suffice it to mention here that almost every kind of nonspecific stress has been used to evoke these latent virus infections into activity: changes in temperature, nutritional upsets, introduction of foreign matter into different parts of the body, irradiation, heterologous infection, various forms of trauma and intoxication, poor husbandry, hormonal disturbances, and so on. It is of great significance that evocation of latent infections by nonspecific stimuli has been observed also with every other class of microbial agents — protozoa, fungi, bacteria, rickettsia — and in many species of experimental animals.

Situations where physiological-biochemical disturbances are far more important than immunological factors in accounting for the difference between infection and disease are common also in diseases of man. The problem of infectious disease in concentration camps during the War has already made us aware of this fact, which can be further illustrated by examples taken from ordinary circumstances of life. It is entertaining in this respect to recall the conditions under which the tubercle bacillus came to be regarded as *the* cause of tuberculosis. In the audience at the meeting of the Berlin Physiological Society where Koch presented his famous report in 1882, practically every person present was certainly infected with virulent bacilli — as almost every one in Europe was infected at that time. Indeed, Koch himself had had a touch of the disease, as shown by the violence of his reaction to tuberculin when he injected the substance into his own arm. Since the tubercle bacillus was then a ubiquitous component of the environment, the factors which converted mere infection into symptoms and destructive pathological changes were the real and practically important determinative causes of tuberculosis. Of these factors we know hardly more today than did Koch in 1882. Tuberculin tests reveal that, even in our most prosperous communities, a very large percentage of the adult population become at some time infected with tubercle bacilli. Yet the morbidity and mortality of tuberculosis have decreased ten- to twentyfold during the past century. It is obvious therefore that while the tubercle bacillus is the specific etiological agent of *infection*, we must look to other factors for a complete understanding of the etiology of tuberculous *disease*.

It may be helpful to illustrate by another example the contrast between infection and disease. In man, the herpes simplex virus is usually acquired during early years. Throughout most of the life of the infected individual, the virus lies latent in the body, without causing any symptom or obvious pathology until "provoked" into activity by some physiological disturbance. As is well known, fever (herpetic) blisters can be elicited by a variety

of nonspecific unrelated stimuli, as different one from the other as menstruation, colds and fevers of various origin, ultraviolet radiation, or eating cheese. Herpetic blisters thus provide a striking example of an infectious disease of man in which, contrary to the original tenets of the germ theory, the living agent of the disease (the germ) may be present all the time in the host, be intrinsic, so to speak, whereas the determinant of the pathological process is some physiological disturbance or some other extrinsic factor of the physicochemical environment (Burnet, 1945).

Toxoplasmosis is a protozoan infection now believed to be extremely common in an inapparent form (Weinman, 1952). Skin tests with toxoplasma antigen suggest that from 30 to 60 percent of the normal adult urban population is infected. Although the immunity mechanisms of the host are unable to bring about the complete eradication of the parasites from the tissues, at least for long periods of time, they are capable under usual circumstances of preventing their multiplication. In the brains of animals the pseudocyst membrane surrounding the parasites persists for several years and is probably responsible in part for the chronicity of the infection. Although severe and even fatal disease processes caused by toxoplasma have been observed in adults, they occur more frequently in the form of abortions, miscarriages, stillbirths, and various defects in the infants who survive. These infections are contracted from the female parent, who is almost invariably in good health during pregnancy and remains a healthy carrier (Weinman, 1952).

The normal microbial flora of the intestinal and respiratory tracts illustrates well the influence of various factors on the ability of tissues to restrain microbial multiplication. In normal animals and man, microorganisms normally present in these areas sporadically gain access to the blood stream, but the transient bacteremias which they cause are of consequence only when an associated abnormality, such as a previously damaged heart valve or a persistent wound, leads to the establishment of a focal in-

fection. As is well known, agents associated with subacute endocarditis are assumed to have often such an origin. In the absence of any abnormalities, the microorganisms are promptly filtered out of the circulation and usually destroyed by reticulo endothelial elements, in the spleen, liver, bone marrow, lung, etc. (Kerby and Martin, 1951). In dogs, clostridia which are potentially pathogenic are normally present in the intestinal tract but do not cause any detectable damage as long as they remain in the gut. Shortly after the application of tourniquet in any part of the body, however, they start multiplying in this area, where they soon reach enormous numbers and produce abundant toxin (Aub *et al.*, 1944).

The cellular and humoral defense mechanisms responsible for the clearing power of normal tissues can be inactivated by all sorts of influences, for example, by radiation, or by mustard gas. The effects of radiation are certainly multiple and complex. Thus, if the dose of x-ray is low enough, it has a fairly specific effect upon the epithelial lining, the crypts and covering of the villi of the small intestine, leaving empty crypts and naked villi exposed to swarms of bacteria. Yet the bacteremia produced under these circumstances rarely leads to an overwhelming invasion and it soon subsides. In contrast, a total impairment of antimicrobial defenses usually follows more intense and total body irradiation of mice. The microorganisms entering the blood stream from the intestinal and respiratory tracts multiply in unrestrained fashion, causing a bacteremia and toxemia which are responsible, at least in part, for the mortality following radiation; this is shown by the fact that many animals can be saved by treatment with antimicrobial drugs (Miller *et al.*, 1950, 1951; Kaplan, Speck, and Jawetz, 1952; Hammond *et al.*, 1954). Clearly, then, the barrier constituted by the epithelium is not the only protection against invasion by microorganisms of intestinal or other origin. There are in the tissues and body fluids multiple antimicrobial agencies probably far more important. We shall have much to say of them in subsequent chapters.

The widespread use of antimicrobial drugs has had unexpected effects which illustrate in a startling manner the complexity of the factors affecting the virulence of the parasites and susceptibility of the host (Finland and Weinstein, 1953). There are many drugs capable of causing almost complete sterilization of the intestinal contents. But whatever the drug used, this antimicrobial effect is usually followed within a few days by the appearance of a new microbial flora which replaces that normally present in the gut. The new microbial population does not necessarily consist of drug-resistant variants of the microorganisms present in the normal intestinal flora; rather, it is usually made up of different microbial species which apparently could not successfully compete with the original flora under normal conditions and had a chance to multiply only after the latter had been eliminated or depressed by antimicrobial therapy. As is well known, therapy with almost any type of antimicrobial drug results not infrequently in a secondary disease process caused by *Candida albicans*. This fungus is probably a constant contaminant of tissues but begins to multiply and manifests its pathogenicity only when the normal *in vivo* environment is disturbed by other debilitating pathological conditions or intensive therapy.

Examples of the disturbance by physiological or physicochemical forces of a state of equilibrium between two living things are not peculiar to the animal world. In nature many bacterial species, called "lysogenic," carry in an inactive form (prophage) one or several bacteriophages potentially capable of causing their lysis. Under ordinary conditions, the prophage is apparently reproduced with each bacterial division without causing any detectable disturbance in the cell. This equilibrium can be upset by a number of nonspecific procedures — for example, by irradiation or starvation of the lysogenic culture or by addition of certain substances to the culture medium — in such a manner that the prophage is converted into active bacteriophage, multiplies abundantly, and causes the destruction of its host cell. Thus, the prophage can become a pathogen for the bacterial cell

that carries it only when the proper kind of stimulus is applied. One might say that the prophage renders the bacterium sensitive to the radiation, or that the radiation renders it susceptible to the prophage, or that both agents are required for the causation of lysis. In fact, it has been shown that, in certain cases at least, the activating effect of radiation can take place only in media of certain composition, thus rendering even more complex the etiological determination of the disease lysis (Lwoff, 1953).

There are likewise many examples of plants which either live in harmony with fungi, bacteria, or viruses or are destroyed by them, depending upon the nature of the physicochemical environment in which the association takes place (Smith, 1952). The root nodule bacteria illustrate well this complex relationship. Under natural field conditions, leguminous plants establish with the nodule bacteria a spontaneous symbiosis which is of great advantage to both. Invasion of the plant by the nodule bacteria is facilitated by the production in the root of a specific exudation at a certain stage in the germination of the plant. Modification of both bacteria and host tissues occurs after invasion, the root nodule constituting in reality a modified root adjusted to the requirements of the bacterial symbionts. Since excessive production of nodules would deprive the plant of its functional root system and thus terminate its existence, the association is regulated by inhibitors produced in the meristems of the root and nodules. But this regulating system itself is under the control of environmental factors, general invasion of the plant by the bacteria taking place if boron is omitted from the soil or culture medium (Thornton, 1952). In this case, therefore, it takes both a nutritional deficiency of the plant (in boron), and the presence of the specific bacteria, to constitute the complete etiology of a change from the symbiotic relationship to the state of disease.

We shall briefly consider, as a last example, the problem of causation of the plant cancers known as crown galls, because the precise knowledge which has been gained of their physiological determinants and chemical basis illustrates in a striking manner

the conceptual difficulties involved in the determination of etiology. It is possible to induce at will characteristic tumors (crown galls) by inoculating certain plants with pure cultures of *Agrobacterium tumefaciens*. Since no other microorganism or substance is known to be capable of causing this pathological reaction, it seems fair to regard *A. tumefaciens* as the specific etiological agent. It has been established, however, that many of the secondary tumors developing on the same plant at sites removed from the initial infection are free of bacteria, and yet can be transferred in series to new plants, or propagated in tissue culture as self-reproducing structures. It is possible also to eliminate the bacterium from the tumor tissue by controlled heating, without affecting the power of autonomous growth of the tumor. Thus, propagation of the cancer can be made independent of *A. tumefaciens*, which was at first its essential etiological agent. It is known, furthermore, that extensive invasion of the plant by the bacterium may take place without resulting in tumor formation. Only plant cells which have been conditioned by certain stimuli associated with wound healing are rendered susceptible to transformation into tumor tissue by the bacterium. The physiological state of the host cells should therefore be considered also as etiological determinant of crown gall. Finally, it can be shown that whereas the normal plant tissue requires indole acetic acid and the cocoanut-milk factor for growth, the self-reproducing tumor tissue does not need these growth factors and indeed can synthesize them (as well, perhaps, as others); it is this biochemical characteristic which permits the cancer to grow profusely and in a completely uncoordinated manner. Thus, at the present state of analysis, the biochemical etiology of the disease appears to reside in an increased synthetic power, but, on the other hand, it takes *A. tumefaciens* to induce the change initially (Braun, A. C., 1952).

Depending upon the specialized interests of the investigator, and the techniques that he choses to use, the primary etiological determinant of crown gall can therefore be regarded as a specific

bacterium, a transmissible cellular change dependant upon a certain physiological state of the cell, or a biochemical disorder. The problem of etiology can be studied at different levels by the bacteriologist, the oncologist, the physiologist, or the biochemist. All points of view are justified and discoveries at any one of the levels of investigation could add to the understanding of pathogenesis and probably lead to some technique of control of the disease.

In the pages that follow, we shall limit our inquiry to the biochemical aspects of the relationships between infection and disease as they are observed in animals and man. There has been no systematic investigation of this field and in no case do the fragmentary facts which are available permit a complete description in chemical terms of the infectious process. For this reason, we shall be compelled to document our discussion by examples taken from many pathological conditions unrelated in microbial etiology, and these examples will be selected not on the basis of their practical importance, but because they serve to illustrate certain points of theory. We shall consider first phenomena which have a bearing on the fate of microorganisms *in vivo*, then the biochemical disturbances resulting from infection which may give rise to the manifestations of disease.

CHAPTER 2

The fate of microorganisms in vivo

THE TISSUES AND BODY FLUIDS AS MEDIA FOR PATHOGENIC AGENTS

The present-day scientific literature dealing with problems of infection often has a quaint mid-nineteenth-century flavor. It describes disease as a conflict between two opponents, one endowed with "aggressive weapons" and guilty of "invasion," the other "mobilizing" various defense mechanisms devised to resist the invader and to protect essential body functions and structures. This mode of thinking, which endows the infectious agent and the host tissue with apparent understanding and means of control over the phenomena of infection, may be useful in guiding the investigator to the discovery of processes established through evolutionary forces. Teleological concepts often fit well biological events and relations between living beings and they have their place in the design of experiments.

It is clear nevertheless that most medical microbiologists feel the need for a type of knowledge that would permit describing the behavior of pathogenic agents in physiological and chemical terms rather than in those used by historians of human warfare. In fact, all textbooks of infectious diseases dutifully begin with chapters devoted to metabolic chemistry. But like the religious convocation which opens political or other lay gatherings, this chapter has little to do with, and is never mentioned in the subsequent proceedings. In practice, metabolic knowledge is not used in the analysis of reactions between host and bacteria. The reason is simply that despite its spectacular advances during the past

three decades, the science of bacterial metabolism and physiology has contributed but little to the understanding of infectious processes.

It is often said that the lag in progress concerning the biochemical aspects of infection is due to the fact that, with a few exceptions, students of microbial metabolism have used in their studies microorganisms never encountered in disease, and have almost completely neglected pathogenic agents. I doubt the cogency of this explanation. Had the biochemists investigated the metabolism of group A hemolytic streptococci, or of anthrax bacilli, or of typhoid bacilli, they would have discovered that these organisms derive energy and carry out organic syntheses by chemical reactions identical with those recognized in lactic acid streptococci, *Bac. megatherium*, or *Escherichia coli*. The genius of metabolic chemistry during our era has been to unravel fundamental processes common to most living things, but it is not in these aspects of metabolism that pathogens differ from saprophytes. Instead, pathogenic behavior seems to depend upon a combination of minor and subtle peculiarities of the infective agents which permit them to survive, multiply, and cause damage in specific *in vivo* environments. And since all animal species, including man, utilize the same fundamental metabolic processes, the determinants of pathogenicity must be sought for in differences so trivial from the point of view of general physiology that they escape the attention of those concerned with the general biochemical phenomena of life.

As one tries to discover a metabolic basis for pathogenicity, it soon becomes apparent that the first question to be answered is not why pathogens can cause disease, but rather why saprophytes do not proliferate as well — or at all — *in vivo*. Tissues and body fluids contain almost every kind of nutrient; they exhibit in different parts of the body a range of oxygen and CO_2 tensions wide enough to provide almost every type of gaseous atmosphere; even H^+ concentrations are found to vary from pH 3.5 to pH 7.5 in various body areas. Since saprophytes are

considered as a rule to be less exacting than pathogens in their growth requirements, it seems at first sight very surprising that they fail to establish themselves and multiply in the animal body. The answer to this riddle will certainly be found in one aspect of the problem which is rarely mentioned and never studied, namely, the very special types of environment which microorganisms find in animal tissues.

Much is known, of course, of the physiological "*milieu intérieur*" maintained approximately constant by the homeostatic mechanisms of the body. It must be realized, however, that this *milieu intérieur* refers only to the extracellular environment in which blood and tissue cells are bathed under normal conditions, and is not the environment in which the infectious process follows its course. For it is certain that immediately after penetrating the body microbial agents find themselves under conditions profoundly different from those termed "physiological."

In vivo, most pathogens as well as saprophytes are rapidly engulfed by various types of phagocytic cells. Some immediately become surrounded by a vacuole; others appear to remain free within the cytoplasm. Hardly anything is known of the inorganic, organic, and gaseous environments that prevail within these phagocytic cells, in or out of the vacuoles, and what little is known adds to the complexities of the problem. There are indications, for example, that following engulfment of certain types of foreign particles and microorganisms the intracellular pH falls to a very low level, at least inside the phagocytic vacuole (see page 37). There is also the most important fact that the various types of phagocytic cells differ in some of their chemical activities (Opie, 1922; Wells, 1925). Recent findings suggest, furthermore, that cellular metabolism is modified during physiologic disorders (natural diabetes or alloxan diabetes), and can be altered by hormones (see page 40). It is clear, therefore, that understanding of the metabolic aspects of intracellular infections — and these are the cause of by far the largest number and most important infectious diseases — will demand a new type

of biochemical knowledge, of both host and parasitic cells, for which techniques of study have not even yet been developed.

The problem is hardly simpler for extracellular infections. It is true that the microorganisms find at first in blood and lymph conditions not too unlike those commonly used in metabolic studies. But this early "physiological" phase of the infection does not last long. The host responds to the presence of parasites by the complex and variable reactions referred to under the generic name of inflammation, and there is no doubt that the inflammatory zone is far different biochemically from the normal tissues and body fluids (Table 1). Whatever the nature of the

TABLE 1

Characteristics of exudates in acute inflammation *

Turpentine injected (ml)	Duration of inflammation (hr)	Sugar (mg/100 ml)		Lactic acid (mg/100 ml)		pH		Cells in exudate (percent)	
		Blood	Exudate	Blood	Exudate	Blood	Exudate	Polymorph	Monos
1.5	0	100	—	—	—	7.5	—		
	19	88	96	11	55	7.5	7.3	85	15
	68	145	117	16	28	7.5	7.3	74	26
2.0	96	81	14	22	136	7.4	6.5	15	85
	115	139	6	38	249	7.4	6.5	2	98
	167	139	35	15	217	7.4	6.5	2	98

Condensed from Menkin, 1940, pp. 78–79.

* The important publications by Frunder (1951, 1953), dealing with the development of an acid reaction in inflammatory areas, came to the author's attention too late to be incorporated in the present monograph.

irritant, inanimate material or living parasite, the inflammatory area is the site of intense glycolytic metabolism on the part of inflammatory and perhaps also of fixed tissue cells: glucose is used up, lactic acid accumulates, and the pH is depressed; there is a decrease in oxygen and an increase in CO_2 tension; alterations in the vascular bed bring about changes in its permeability and in blood flow (Schade, Neukirch, and Halpert, 1921; Kempner and Peschel, 1930; Rich, 1936; Kelley *et al.*, 1938; Menkin,

1940, 1950; Frunder, 1953). Furthermore, activation of plasma proteases and autolytic processes following necrosis result in the release of a variety of tissue constituents and breakdown products. All these happenings are modified qualitatively and quantitatively by the type of infectious agents that bring them forth, by the physiological state of the infected host, and by the particular anatomical location in which inflammation occurs.*

It must be kept in mind, on the other hand, that there exist in the different tissues and body fluids a host of substances which can under the proper conditions exert toxic effects on microorganisms, and which tend to accumulate, or be activated, in inflammatory and necrotic areas — particularly as a result of reactions associated with immunity and allergy (see pages 42–57).

The physicochemical characteristics of the tissues have not only quantitative effects on the survival and multiplication of microorganisms, but also qualitative effects on their biological properties (see pages 58–64). Relatively small differences in the physicochemical environment can profoundly influence phenotypic expressions like the production of toxins or the accumulation of surface antigens, as well as heritable characters resulting in marked changes in the microbial populations. In other words, the microorganisms involved in the production of disease — whether multiplying intracellularly, or in an inflammatory area, or in the midst of necrotic tissues — differ profoundly from the forms of the same species grown *in vitro* under conditions totally unlike the complex and changing environment created by the infectious process.

It is in the subtle complexities of the *in vivo* environment that resides the explanation of the extraordinary specific character of the phenomena of pathogenicity. The pathogenic potentialities of a given microorganism — including its ability to survive and

* Many important aspects of the biochemistry of inflammatory areas are discussed in the proceedings of an international symposium recently published under the title *The mechanism of inflammation*, edited by G. Jasmin and A. Robert (Acta Inc., Montreal 1953).

multiply *in vivo* as well as to cause pathological disturbances — can hardly be revealed by studying its behavior in a stereotyped medium selected for the convenience of biochemical analysis. An understanding of pathogenesis demands that the phenomena of growth and of toxicity be observed under the highly specialized environment characteristic of each host, and of each phase of the infection.

In order to render more apparent the absolute necessity of considering the local tissue environment in the metabolic study of infectious processes, it may be worth while to outline in very broad terms a few special problems which can be apprehended only in the light of this point of view.

Let us examine, for example, the behavior *in vivo* of the strains of viruses or bacteria which are used as living vaccines for immunization against certain virulent infections. In general, the strains of yellow fever virus (17 D), Newcastle virus (NDV–B), *Myc. tuberculosis* (BCG), *B. anthracis* (Pasteur's first and second vaccine), *B. pestis* (Girard and Otten strains), *Br. abortus* (19), etc., which are used to protect against yellow fever, Newcastle disease, anthrax, tuberculosis, plague, brucellosis, etc., are referred to as "avirulent" because they cannot cause fatal disease in experimental animals or man under usual conditions. There is no doubt, however, that these "avirulent" microorganisms do multiply extensively in the tissues and body fluids of the vaccinated hosts; it is for this reason that Pasteur wisely referred to them as "attenuated" rather than "avirulent." Indeed, it is by virtue of their ability to cause a definite, if abortive, disease that they can elicit immunity against virulent infection. Thus, the attenuated "avirulent" forms used for vaccination differ from the virulent parent strains only by the fact that their proliferation *in vivo* is not extensive or prolonged enough to lead to overt, fatal disease.

A similar situation is encountered when one compares the behavior *in vivo* of the bovine and human types of virulent tubercle bacilli. Injection of very few bacilli of either type into

guinea pigs or mice results in a fatal outcome, but it is only the bovine bacillus which can cause progressive tuberculosis in cattle or rabbits. This does not mean, however, that bacilli of the human type fail to find in the tissues of cattle or rabbits growth factors and an environment suitable for their multiplication. In fact, quantitative bacteriological studies have shown that during the first few days following infection the human bacilli multiply very rapidly and invade all organs in these animal species, just as do the bovine bacilli (Lurie, 1928, 1953). But whereas the multiplication of the latter continues and gives rise to progressive disease, that of the former soon stops and is rapidly followed by massive bacillary destruction (Table 2). Clearly, tuberculous in-

TABLE 2

Number of colonies recovered from organs of rabbits infected with human tubercle bacilli

Time after injection	Infective dose (per kg)			
	0.001 mg		0.1 mg	
	Liver	Spleen	Liver	Spleen
1–48 hr	1.5	4.5	35	100
1 wk	3	14.3	283	1000
2 wk	43	968	350	—
4 wk	47	2344	333	455
8 wk	0	69	4	8
16 wk	0	0	0	0

Condensed from Lurie, 1928, pp. 158, 166, 167.

fection brings about in the tissues of cattle and rabbits a change which is more inimical to the human than to the bovine bacilli. It is in terms of that particular change that the metabolic characteristics responsible for the differences in virulence between human and bovine bacilli must be defined.

Present metabolic knowledge is not sufficient to provide a biochemical basis for these differences in virulence, but some biological facts can help in formulating an approach to the problem. In some cases the difference in outcome of the infections caused by the virulent and attenuated strains of a given microbial

agent is determined by the initial rate at which these agents multiply in certain particular organs of the host under consideration. Yet this does not mean that intrinsically, the vaccine strain has a longer replication time than the virulent strain; in fact, both multiply at the same rate when injected in certain environments. Thus, in the case of the Newcastle disease virus, the virulent and vaccine strains multiply at the same rate in extraneural tissues of the chicken (blood, lung, rectum, and spleen), but the former multiplies much more rapidly than the latter in the brain and this difference can be recognized very soon after infection. In the chick embryo, both strains multiply equally fast in the cells lining the allantoic sac, but they display marked differences in other organs (Karzon and Bang, 1951; Liu and Bang, 1953) (Figs. 1 and 2).

Even more striking differences have been recognized between virulent (viscerotropic) and attenuated (neurotropic) strains of yellow fever virus. If monkeys are inoculated peripherally with a mixture of the two strains, the viscerotropic virus multiplies faster than the neurotropic, whereas the reverse is true if the

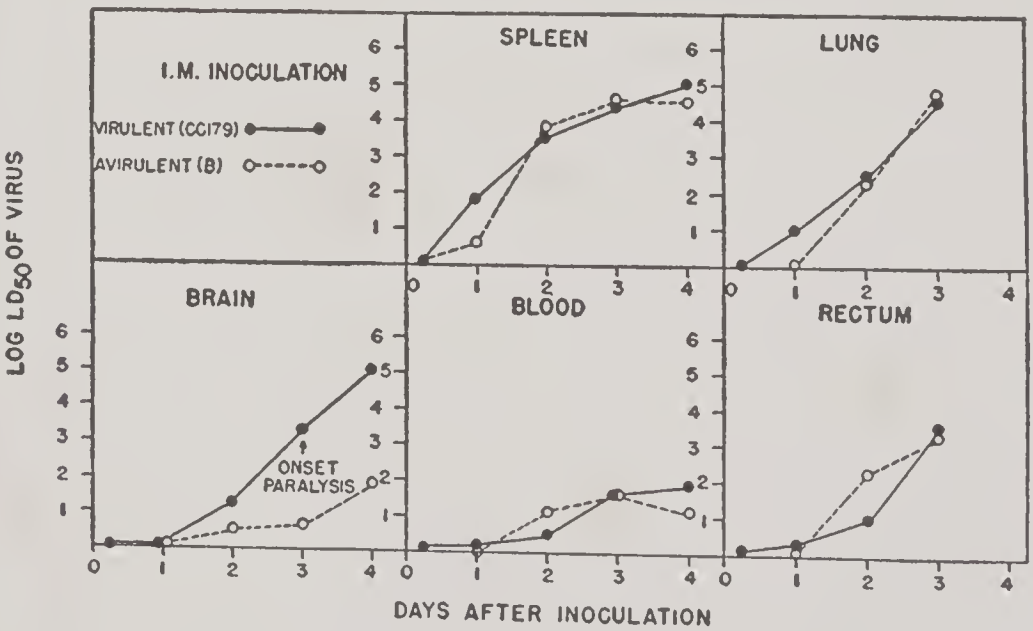

Fig. 1. Rates of multiplication of two Newcastle disease viruses of unlike virulence, in various animal tissues (Karzon and Bang, 1951).

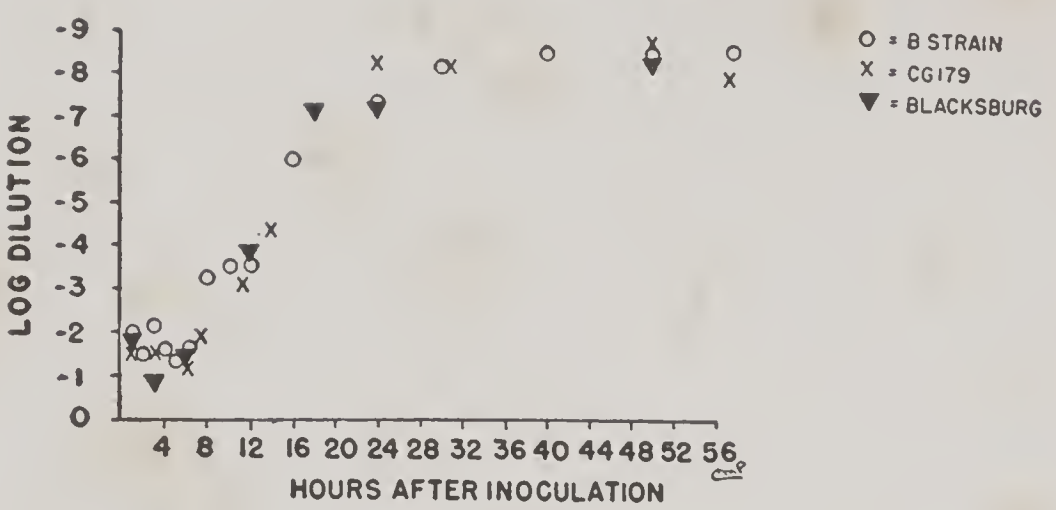

Fig. 2. Rates of multiplication of three Newcastle disease viruses of unlike virulence in chick embryos following allantoic sac inoculation (Liu and Bang, 1953).

mixture is injected into the brains of mice (Theiler, 1951). It is clear, therefore, that the reasons for the differences in virulence between the vaccine and virulent strains of the viruses under consideration must be looked for in differences in the normal biochemical environment of certain specific organs (see also Habel, 1954).

Quantitative bacteriological experiments with virulent and attenuated (so called avirulent) BCG strains of tubercle bacilli in the mouse have revealed phenomena of a similar nature. However small the dose of BCG bacilli injected, these organisms invade the various organs and proliferate in them. However large the dose, on the other hand, they multiply more slowly than do bacilli of the virulent strains, even when extremely small inocula of the latter are used. These findings are illustrated in Fig. 3, which reveals furthermore that marked differences also exist between various strains of BCG (see page 97). Yet virulent and BCG strains multiply at the same rate under aerobic conditions *in vitro* (Pierce and Dubos, unpublished).

It is possible that in some cases the initial rate of multiplication in the tissues of a given animal is the same for two strains of unequal virulence but that the environment caused by the inflammatory reaction is more inimical to one strain than to the

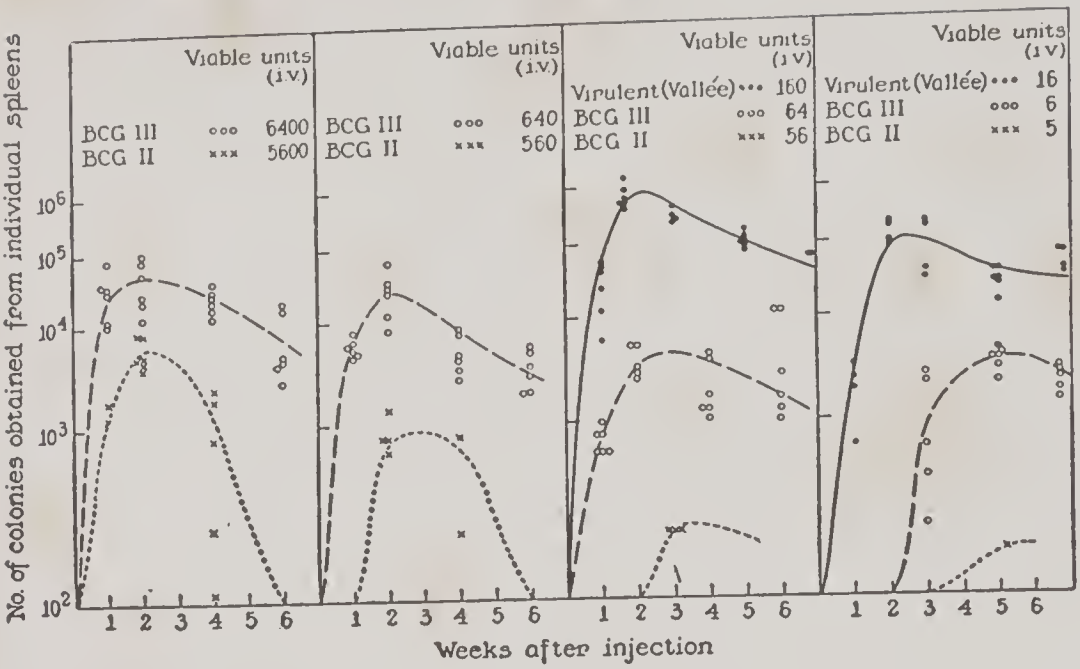

Fig. 3. Multiplication in the mouse of one virulent and two attenuated strains of bovine tubercle bacilli (Pierce and Dubos, unpublished).

other — inhibition of growth of the form attenuated for that particular animal species resulting either from the local accumulation of toxic substances or from other unfavorable metablic conditions. The difference in virulence between the human and the bovine tubercle bacilli toward cattle and rabbits seems to pertain of this type of mechanism (Table 2). The findings illustrated in Fig. 3 and Table 2 show that the mycobacterial population in the spleen begins to decrease with all strains after a very few weeks. Several lines of evidence, which cannot be reviewed here, strongly suggest that the more virulent the strain, the more resistant it is to the growth-inhibitory conditions and substances that prevail at the site of the lesion as a result of inflammatory and immune processes.

The biochemical changes which occur in tissues during infection are relevant not only to the understanding of problems of pathogenesis, but also to the practical problems of prophylaxis and therapy. Phagocytes, antibodies, or antimicrobial drugs cannot exert their protective effect unless they come into contact with microorganisms under the proper physicochemical condi-

tions. Antibodies do not seem to function within intact phagocytic cells (Rous and Jones, 1916; Shaffer, Kucera, and Spink, 1953) and do not react with homologous antigens at acid reaction. Phagocytosis is much more effective on body surfaces than in edema fluid (Wood, 1951; Roth, 1950).

It is well known also that antibacterial drugs which are highly effective in the extracellular environment may fail to affect microorganisms within phagocytic cells (Rous and Jones, 1916; Shaffer, Kucera, and Spink, 1953; Mackaness, 1952; Mackaness and Smith, 1953; Suter, 1952). Others which are able to prevent the establishment of infection or its spread to new areas of the body, are completely inactive in certain types of lesions (empyemas, abcesses, necrotic areas, etc.) (see page 112). These discrepancies are to be traced in part to the fact that drugs are screened *in vitro* under "physiological" conditions (pH approximately neutral, absence of breakdown products of tissues and of unusual metabolites, adequate aeration, low oxygen tension, etc.). In other words, they are selected on the basis of their ability to function in the physiological environment which prevails extracellularly *in vivo* before phagocytosis, inflammation, and necrosis have occurred. It is probably for this reason that they are often so ineffective in established lesions, where conditions are so different from the physiological. New types of useful screening tests could well be developed if more knowledge were available of the varied environments in which therapeutic agents are called to function *in vivo*.

It seems unnecessary to develop further the argument that any metabolic analysis of the infectious process must be placed on an ecological basis. In many respects, the problems of medical microbiology demand the kind of conceptual approach that was formulated by S. Winogradski (1924, 1925) for the study of soil microbiology. As will be recalled, Winogradski pointed out the fallacy of attempting to derive conclusions concerning the role of microorganisms in soil processes from the study of their behavior in artificial culture media under conditions designed for the con-

venience of microbiological and chemical experiments. He advocated the development of new techniques that would permit the study of microbial activities in an environment as similar as possible to that found in the soil.

We need not consider here the obvious difficulties and limitations of this approach to the problems of soil microbiology. The difficulties are even greater and more apparent in the study of infectious processes. But granted that it is not practically possible to define in chemical terms the continuously changing environment in which infection proceeds, we must recognize nevertheless that no metabolic analysis of infectious disease is possible until an ecological concept is introduced to formulate the problem. It is because this ecological concept has been lacking almost completely heretofore that bacterial biochemistry has contributed so little to the understanding of pathogenesis.

Winogradski pointed out that each fragment of soil constitutes a biosphere with its own chemical and biological peculiarities. The study of diseased tissue by histological techniques leaves no doubt that even within the same organ there can occur many different types of lesions which provide for the infective agent a whole range of metabolic conditions. It would be impossible naturally to provide a complete account — in space and in time — of the biochemical environment during the whole course of each type of infection. But it is possible to study some of the factors which play a part in determining quantitatively and qualitatively the fate of microorganisms *in vivo*, as well as the reaction of tissues to their presence. It is the interplay of these factors which decides whether infection can progress far enough to express itself in overt disease.

At the risk of much artificiality, and merely for the sake of convenience, we shall discuss separately and in succession — as if they were distinct entities — the factors which favor microbial proliferation, the tissue agencies which are lethal or toxic for microorganisms, and finally the effect of the environment on the qualitative characteristics of microorganisms.

THE PROLIFERATION OF MICROORGANISMS *IN VIVO*

The field of plant pathology provides many examples where host-parasite relationships could probably be analyzed in terms of nutritional requirements of the parasite and availability of nutrients in the host. These relationships are of course extremely complex, and they depend not merely on the presence or absence of a given metabolite in plant tissues, but also on the proper balance between different biochemical factors. This "balance hypothesis of parasitism" has been recently presented in a most stimulating review (Lewis, 1953).

In the field of animal pathology, there is no case where the metabolic behavior of infectious agents, or the nature of the *in vivo* environment, is sufficiently well understood to afford a chemical explanation for the property of virulence. Among fully encapsulated pneumococci of one given serological type, for example, some are highly virulent for mice and others are not; similar unexplained differences occur among streptococci of group A in the M phase, or among mouse typhoid bacilli endowed with a full panoply of surface antigens. Nor is there any metabolic explanation for the fact, already referred to, that the rates of multiplication of the virulent strains of yellow fever or Newcastle virus are greater than those of their attenuated (vaccine) counterparts in certain hosts but not in others, or that the bovine strains of tubercle bacilli reach in rabbits and cattle a higher population level than do the human strains (see page 18).

While metabolic chemistry has remained so far silent with regard to the problem of virulence (see, however, pages 67 and 96), it has thrown some light in a few cases on the reason why parasites may remain dormant in the tissues for long periods of time, and then start multiplying as conditions change. This phase of medical microbiology was heralded by Pasteur's discoveries that oxygen is an essential factor for the growth of the anthrax bacillus, and an inhibitor of clostridia (reviewed in Dubos, 1950*b*). Among aerobic organisms, there is none which reacts more dra-

matically to the presence of oxygen than do tubercle bacilli. *In vitro*, the yield of bacillary growth is enormously increased when oxygenation of the culture is facilitated by incubation on surfaces, by agitation, or by forced aeration. *In vivo*, the bacilli do not multiply in closed caseous areas and, in fact, they progressively disappear from these lesions. In contrast, they reach enormous numbers in the caseous matter lining the inner part of open cavities, or in any cavity with a patent opening into a bronchus, showing that inhibition of growth within the closed lesions is due in part to oxygen deficiency (reviewed in Canetti, 1946) (see page 108).

It is well known that spores of *Cl. tetani*, *Cl. welchii*, or other clostridia can persist for years in the tissues without causing any symptom of disease or even multiplying, whereas they are often brought into activity by nonspecific stimuli such as trauma, surgical intervention, or local infections. It is probable that the various circumstances which permit germination of the spores and multiplication of the bacilli have at least one factor in common — namely, their ultimate effect in causing the oxidation-reduction potential to fall to a critical low level (Fildes, 1929). This can be brought about through an increased local consumption of oxygen (by a contaminating aerobic microbial flora or by inflammatory cells), or through interference with oxygenation by disturbance of blood flow, or also perhaps through the reducing action of the –SH groups which are unmasked during the denaturation of proteins resulting from necrosis.

As many pathogens have complex nutritional requirements, it readily comes to mind that their multiplication *in vivo* might be limited by the deficiency of the tissues in certain essential growth factors. Only during recent years has some evidence been obtained in favor of this hypothesis. It has been found, for example, that the multiplication of certain viruses is somewhat inhibited in animals fed diets deficient in protein and in some of the vitamins (reviewed in Clark *et al.*, 1949). Results in accord with these findings have been obtained *in vitro*. Thus, whereas poliomyelitis

virus does not multiply in human tonsillar tissue maintained in Simm's solution containing serum ultrafiltrate, viral multiplication takes place if the same tissue is cultivated in the more complete synthetic medium 199 (Smith, Chambers, and Evans, 1950; Franklin *et al.*, 1953). Similarly, psittacosis virus survives without multiplication in tissue cultures maintained in nutritionally deficient media, but starts multiplying upon addition of hydrolysate of lactalbumin to the nutrient fluid (Hare and Morgan, 1954). Needless to say, the influence of vitamin and amino acid deficiency on virus production may be extremely indirect and result from some disturbance of tissue metabolism rather than from an effect on the virus itself.

In the case of bacteria, inhibition of growth *in vivo* because of lack of an essential nutritional factor has been recognized only in artificial laboratory models. Certain mutants of *Salmonella typhi* and *Klebsiella pneumoniae* require for growth one or another of several amino acids, purines, pyrimidines, or vitamins. When the purineless and PABA-less mutants were tested in mice, it was found that they were unable to produce disease unless proper amounts of either purines or PABA were injected into the animals or added to their diets; obviously, it was the lack of these substances in the tissues in a state available for microbial utilization which prevented the microorganisms from multiplying and manifesting their potential pathogenicity (Tables 3, 4, and 5). In con-

TABLE 3

Specific enhancement of infection caused by metabolic mutants of *S. typhi*

Infective organism	Deaths out of 20 mice receiving		
	Hypoxanthine *	PABA *	Arachis oil
PABA-less	0	11	0
Purineless	18	0	0
Wild type	16	16	13

After Bacon, Burrows, and Yates, 1951.

* 4 mg resuspended in 0.25 ml arachis oil, injected i.p.

TABLE 4

Effect of dietary PABA on virulence of PABA-less mutant of *S. typhi*

Infective dose ($\times 10^6$)	PABA (5 mg) fed on days before challenge					Deaths out of 20 mice
	5	4	3	2	1	
400	0	0	0	0	0	3
100	0	0	0	0	0	0
100	0	0	0	0	+	16
100	0	0	+	+	+	18
100	+	+	+	+	+	20

From Bacon, Burrows, and Yates, 1951.

TABLE 5

Virulence of biochemical mutants of *Kl. pneumoniae*

Treatment of mice (i.p.)	Infective dose (i.p.) ($\times 10^4$)	Deaths in groups of 10 mice infected with following strains:			
		Purine-less	Uracil-less	Amino acid-less	Wild type
None	1	0	10	10	10
None	10	0			
None	800	0			
Hypoxanthine 50 mg	10	3			
Purine pool * 50 mg	800	10			

After Garber, Hackett, and Franklin, 1952.

* 15 mg hypoxanthine, 3 mg xanthine, 3 mg adenine, 3 mg guanine.

trast, the amino acid-less and pyrimidine-less mutants multiplied as readily *in vivo* as the parent strains, evidently because amino acids and pyrimidines were available for them in the mouse tissues (Bacon, Burrows, and Yates, 1951; Garber, Hackett, and Franklin, 1952).

Of more direct relevance to the problem of natural disease is

the recent discovery that malaria parasites, which require PABA for growth, fail to produce progressive infection if the diet of the infected animal is deficient in this vitamin. Thus rats are resistant to *Pl. burghesi* if fed on a milk diet, and susceptible if fed on a more complex food mixture (Tables 6 and 7). This is not

TABLE 6

Percentage of erythrocytes parasitized by *Pl. berghei* in rats on different diets

Day after inoculation	Normal diet	Milk	Milk + PABA 1/1000
7	1–5	0–0	2.5–4
12	6–20	0.5–0	8–10

After Hawking, 1953.

TABLE 7

Development of *P. berghei* in suckling rats receiving p-aminobenzoate (P.A.B.) by different routes

Treatment	Mean parasitemia * after (days)			
	4	5	6	7
Controls	4.6 (3/8)	13 (4/8)	51 (6/8)	126 (8/8)
Babies inoculated with 0.1 mg P.A.B. i.p.	2,000 (8/8)	3,400 (8/8)	—	2,800 (8/8)
Mother smeared with P.A.B.	1,250 (8/8)	2,400 (8/8)	—	3,100 (8/8)
Mother inoculated with 10 mg P.A.B. i.p.	2,000 (8/8)	4,000 (8/8)	—	3,500 (8/8)
Young rats (50 gm) on stock diet		1,000 (4/4)	3,000 (4/4)	

From Hawking, 1954.

* Mean parasitemia is expressed as the geometric mean of the number of parasitized erythrocytes per 20,000 cells. The figures in parentheses indicate the proportion of animals showing parasites.

due, as was naturally first thought, to the fact that milk contains a substance favoring resistance to infection, but instead to the lack of PABA in the food. Indeed, rats can be rendered susceptible to the plasmodia by being fed a milk diet supplemented with the vitamin or with folic acid. Similarly, monkeys on a milk diet are resistant to infection by *P. knowlesi* or *P. cynomolgi*, but become fully susceptible when PABA is added to the milk in amounts of 0.5 mg per kilogram body weight per day. As human infants under six months of age are relatively immune to malaria in many tropical countries, it appears likely that the findings with rats and monkeys may be applicable to human beings as well (Hawking, 1953, 1954).

In addition to substances essential for microbial proliferation, there are probably others which enhance infection by increasing either the rate of multiplication, or the total amount of growth. It is seen in Tables 8 and 9, for example, that staphylococci and

TABLE 8

Effect of various metabolites on the multiplication of staphylococci at acid reactions

Substance added to the medium (final concentration 0.025 M)	Growth * after 18 hrs.' incubation (37° C) at indicated pH			
	5.3	5.5	5.8	6.9
0	−	++	++++	+++
Acetic acid	−	−	−	+++
Propionic acid	−	−	−	
Butyric acid	−	−	−	
Lactic acid	−	+	++	++++
Fumaric acid	−	−	+++	++++
Pyruvic acid	++	++++	++++	+++
α-Ketoglutaric acid	−	+	+++	++++
β-Hydroxybutyric acid	+	++	+++	++
Glutamic acid	−	++	++	++++
Dihydroxyacetone		++++		

From Dubos, 1953*b*.

* Recorded according to an arbitrary scale (visual observation) from − (no growth) to ++++ (maximum growth).

TABLE 9

Effect of various metabolites on the multiplication of tubercle bacilli at acid reactions

Substance added to the medium (final concentration 0.025 M)	Growth * after 4 days' incubation (37° C) at indicated pH					
	4.5	5.2	5.5	5.8	6.4	6.9
0	−	−	++	++	+++	++
Acetic acid	−	−	−	−		+++
Propionic acid	−	−	−	−		+++
Butyric acid	−	−	−	−		+++
Lactic acid	−	−	−	−	+++	++++
Fumaric acid	−	−	++++	++++	++++	++++
Pyruvic acid	−	−	−	++	++++	+++
β-Hydroxybutyric acid	−	−	−	+++	++++	++
α-Ketoglutaric acid	−	++	++++	++++		++
Glutamic acid	−	+++	++++	++++	++++	++++
Dihydroxyacetone	−	++	+++	+++	+	−

From Dubos, 1953*b*.

* Recorded according to an arbitrary scale (visual observation) from − (no growth) to ++++ (maximum growth).

tubercle bacilli grow readily *in vitro* at acid reactions if certain polybasic acids or keto acids are added to the culture medium (Dubos, 1953*a*, *b*; Weiss and Dubos, 1954, unpublished observations). Indeed, it is shown in a recent publication that suspensions of *Escherichia coli* which had apparently been "sterilized" by certain treatments (as determined by failure of growth in conventional culture media) can yield large numbers of viable cells when incubated with various metabolites of the tricarboxylic acid cycle (Heinmets, Taylor, and Lehman, 1954) (Table 10).

It seems legitimate, therefore, to consider the possibility that certain metabolic disorders increase susceptibility to infection by making available to infectious agents substances not normally present in any significant amount in the tissues and body fluids. Although convincing evidence of this possibility has not yet been obtained, there are a few experimental findings which appear compatible with it, and which may provide support for it on

TABLE **10**

Restoration of viability by the use of metabolites in *Escherichia coli*, strain B/r, which have been "killed" by chemicals and heat

Substrate Number	Mode of treatment *	Number of viable cells				
		Heat	Chlorine	H_2O_2	"Zephiran" chloride	Ethyl alcohol
	Before treatment	3.1×10^6	1.1×10^6	1.4×10^7	3.5×10^6	3.5×10^6
	After treatment	0	0	8.0×10^1	0	0
	Substrate	Treated samples incubated with substrate at 37° C for 24 hr				
1	Buffer (pH 7)	0	0	0	0	3.1×10^3
2	Sodium pyruvate	0	0	8.0×10^1	0	4.4×10^4
3	Oxalacetic acid	2.3×10^3	3.2×10^3	3.0×10^3	1.4×10^3	2.2×10^4
4	Sodium acetate	0	0	1.6×10^3	0	3.6×10^4
5	Sodium citrate	1.3×10^4	1.1×10^4	1.4×10^5	$> 1.5 \times 10^6$	4.1×10^1
6	*cis*-Aconitic acid	4.0×10^1	0	—	1.5×10^5	$> 1.5 \times 10^6$
7	Isocitric acid	0	0	—	2.8×10^5	3.1×10^4
8	Lactic acid	6.4×10^3	3.4×10^3	0	$> 1.5 \times 10^6$	4.4×10^4
9	Malic acid	8.0×10^1	1.5×10^4	5.8×10^3	3.6×10^3	5.0×10^4
10	Succinic acid	1.4×10^2	0	0	0	1.4×10^5
11	α-Ketoglutaric acid	2.1×10^2	0	5.1×10^2	0	$> 1.5 \times 10^6$
12	Sodium fumarate	4.1×10^1	0	0	0	2.2×10^4
13	Mixture of 2, 3, 4	1.0×10^2	0	—	2.5×10^1	5.1×10^4
14	Mixture of 3, 4, 6	0	2.4×10^4	—	6.0×10^3	2.8×10^4
15	Mixture of 7, 10, 11	0	0	—	0	1.1×10^5
16	Mixture of 2 to 12	2.1×10^6	2.5×10^5	1.1×10^6	$> 1.5 \times 10^6$	$> 1.5 \times 10^6$
17	Nutrient broth	0	0	—	0	—

From Heinmets, Taylor, and Lehman, 1954.

* Contact time of bacteria with heat and various chemicals (1) heat, 30 sec at 71° C; (2) chlorine (1 ppm), 6 min; (3) hydrogen peroxide (0.3 percent), 25 min; (4) ethyl alcohol (20 percent), 10 min; (5) "zephiran" chloride ($1:1 \times 10^5$), 6 min.

further analysis. We have found, for example, that animals whose metabolism has been disturbed by treatment with small doses of thyroid extract or dinitrophenol, or by adding to the diet certain substances like citrate, pyruvate, etc., particularly in association with glycerides of short-chain fatty acids, become much more susceptible to infection with tubercle bacilli, staphylococci, and Friedlander bacilli (Table 11). It has been demonstrated also that the survival of mice infected with *S. typhimurium* can be shortened by treating the animals with substances which poison the Krebs cycle (malonate, arsenite, fluoroacetate) or by daily injection of some of the metabolites, citrate and succinate

for example, assumed to accumulate in the tissues if this cycle is interfered with (Table 12) (Berry and Mitchell, 1953).

TABLE 11

Effect of nutritional and metabolic factors on susceptibility of mice to tuberculosis

Diet *ad lib.* *	Infection (ml of culture)	Deaths (out of 10) at indicated days after infection			
		11–20	21–30	31–40	41–50
A	0.01 Vir.				5
B	0.01 Vir.	2	4	3	
C	0.01 Vir.		4	2	2
D	0.01 Vir.		5	4	1
E	0.01 Vir.	2	5	1	1
A	0.2 BCG				
B	0.2 BCG	1	2		4

Dubos, Pierce, and Smith, unpublished observations.

* A, normal diet (flour, milk, salts); B, normal diet + 0.05 percent thyroid extract; C, normal diet + 0.1 percent dinitrophenol; D, flour + 5 percent skim milk + 20 percent fat; E, flour + 5 percent skim milk, + 20 percent fat + 6 percent citrate.

TABLE 12

Effect of treatment with sodium citrate or succinate on survival of mice infected with *S. typhimurium*

Time after infection (hr)	No. of injections *	Survivors, of groups of 5 treated with:		
		Saline	Succinate	Citrate
3	3	5	5	4
5	5	5	5	3
7	7	5	5	0
24	8	5	1	0
58	8	5	0	0
118	8	3	0	0
130	8	1	0	0
142	8	0	0 †	0 †

After Berry and Mitchell, 1953.

* Citrate (10 mg) or succinate (20 mg) injected hourly.

† All of treated, noninfected mice still surviving and healthy at end of experiment.

Granted their great interest, all these experiments are obviously too simple and preliminary to justify any attempt at analysis of the biochemical events *in vivo* which are responsible for increase in susceptibility to infection. It has been convincingly shown, for example, that poisons which interfere with the Krebs cycle inhibit the multiplication *in vivo* of certain viruses, a finding which illustrates that metabolic disturbances of the tissues can modify the course of infection not only by changing qualitatively and quantitatively the nutrients available to the microorganisms, but also by altering host resistance in unknown ways.

THE FATE OF MICROORGANISMS WITHIN PHAGOCYTES

Let us emphasize at the outset that phagocytosis is not necessarily a protective mechanism against infection. It is true that phagocytes help the body to rid itself of foreign particles, of certain microorganisms in particular. But on the other hand all viruses and rickettsia, as well as many species of bacteria and protozoa, are either obligatory or facultative intracellular parasites. Not only do they multiply within phagocytic cells, but these protect them from attack by antibodies or drugs (Rous and Jones, 1916; Shaffer, Kucera, and Spink, 1953; Suter, 1952*b*; Mackaness, 1952; Mackaness and Smith, 1953). In fact, the theory that phagocytosis facilitates the spread of infection was the first to find favor among the 19th-century pathologists. They had observed the phenomenon of phagocytosis long before Metchnikoff rediscovered and named it, but they believed that the phagocyte provided for the infectious agent food and transportation through the tissues (reviewed in Cameron, 1952, p. 195).

Had Metchnikoff been trained in medicine, he might also have accepted as a matter of course the view that most parasites survive and even multiply in the intracellular environment. But it was as a general biologist, and not as a pathologist, that he first became interested in the problems that led him to formulate the phagocytic theory of immunity. Metchnikoff regarded the uptake of particulate matter by cells as the most primitive and funda-

mental mode of nutrition. He believed that while this activity was still used by unicellular organisms for procuring food, it persisted as a vestige in the specialized cells of more highly organized living creatures. According to his own account, it was with these thoughts in mind that in 1882, while working at the Marine Laboratory in the Straits of Messina, he had the sudden inspiration that the uptake of particles could be used by living things as a means of defense against intruders. For the ameboid cells of mesodermic origin, found in the blood and body fluids and scattered throughout the tissues, he invented the name of "phagocytes" (devouring cells) in order to recall the primitive nutritional aspect of their activities (Metchnikoff, 1905).

The engulfment of particles and microorganisms is facilitated by the fact that phagocytes are endowed with special properties which enable them to perceive exceedingly subtle changes in the surrounding medium. There is some indication that local increase in concentrations of potassium can bring about the transformation of resting wandering cells into active macrophages without preceding division. Thus, many forms of injury, by releasing K^+, could stimulate phagocytic activity in the absence of other signs of inflammation (Tompkins, 1953; Tompkins and Grillo, 1953). Most extensively studied has been the effect of a variety of substances — including microbial components and products of tissue breakdown — on the movements of migratory leucocytes. As is well known, some substances exert on leucocytes a positive chemotactic effect, whereas others inhibit their migration. This aspect of the problem cannot be discussed here.

Both *in vitro* and *in vivo*, phagocytosis takes place most readily when leucocyte and parasite come into contact on the proper kind of surface (Wood, 1951; Wood, Smith, and Watson, 1946; Roth, 1950). Surface phagocytosis is certainly of very wide occurrence in the tissues and accounts for the tremendous "clearing" power of many organs, a property which allows the rapid removal of most microorganisms from the circulation under normal circumstances. In contrast, when both the leucocyte and

the parasite are immersed in a fluid phase, phagocytosis is inefficient and often requires the presence of specific antibody.

The effect of the physical nature of the environment on the phagocytic power of leucocytes is of special interest for the pathogenesis of certain bacterial diseases. It is probable, for example, that the reason why exposure to pneumococci or streptococci results only infrequently in progressive infection, even in nonimmune individuals, is that these bacteria are readily disposed of by surface phagocytosis. If, however, bacteria reach unharmed a fluid environment such as that caused by edema, phagocytosis is either prevented or sufficiently retarded, thus permitting microbial proliferation to occur. The experimental infection of the mouse with virulent pneumococci provides a striking illustration of these phenomena. With mouse-virulent strains, it is possible to establish a fatal peritonitis by injecting one or a very few living pneumococci intraperitoneally. In contrast, it takes many thousands or millions of pneumococci to establish infection by the intravenous route, and it is practically impossible to do it by causing the animals to inhale bacterial suspensions (unless pulmonary edema has first been produced by some irritating agent like mustard gas). It seems likely that this difference in behavior is to be traced to the fact that the pneumococci introduced either intravenously or by inhalation rapidly fall prey to surface phagocytosis, whereas those injected into the peritoneal cavity are for a while protected from the phagocytic cells by the dilution fluid and exudate.

As already mentioned, the word phagocyte was devised by Metchnikoff to convey the concept that the leucocyte was capable of "devouring" the parasite which it had engulfed. The word is picturesque, but the concept far from clear. Most parasites thrive within the cells that phagocytize them and it is not at all certain that those that die are "devoured" by these cells. Let us now briefly consider the fate of microbial parasites in the intracellular environment, whatever the circumstances under which phagocytosis took place.

It is an extraordinary fact that certain bacteria die within a very few minutes after having been phagocytized. This can be recognized through the failure to obtain cultures of them on artificial culture media, and by seeing the organisms disintegrate rapidly within the phagocytes (Wood, 1951; Rogers and Tompsett, 1952; Wilson, 1953). Moreover, death and disintegration are found to occur equally fast with delicate organisms like pneumococci or group A streptococci and with microbial species like Friedlander bacilli, staphylococci, and group D streptococci which are usually regarded as much more resistant to injurious agents.

Metchnikoff, and many investigators after him, attributed this lethal effect to a variety of enzymes which they called "cytases" or "digestive ferments," and which were assumed to be capable of digesting the microorganisms within the phagocytic vacuole (Metchnikoff, 1905). It is true that leucocytes contain a large variety of proteases, lipases, esterases, saccharidases, oxidases, and so on — indeed the whole battery of enzymes present in any cell capable of leading an independent life (Opie, 1922). So far, however, there has not been published any valid evidence that these enzymes can exert directly a bactericidal effect on any microbial species (see page 45). The known enzymes can merely attack some of the various components of dead microbial agents, or surface components of living cells not essential to viability. There is only one exception to this statement. Leucocytes, like many other animal cells, produce an enzyme of the lysozyme type which has bactericidal and lytic properties similar to or identical with those of the classical lysozyme isolated from egg white. Lysozyme seems to be particularly abundant in granulocytes and may well play some part in their ability to kill and lyze certain microorganisms (see page 46). Other factors, however, must also come into play, for several microbial species which are resistant to lysozyme succumb rapidly in the intracellular environment of the phagocytes.

As already pointed out, the death of microorganisms within

leucocytes may occur very rapidly — the time required is less than one hour, often a matter of minutes. It is known on the other hand that the phagocytosis of certain types of material is immediately followed by an intracellular burst of acidity which brings the pH of the phagocytic vacuole to a very low level. Metchnikoff first recognized this phenomenon by following the change of color of litmus particles within the cell (Metchnikoff, 1905). Subsequent studies with a variety of pH indicators have confirmed his findings (Rous, 1925; Pulcher, 1927; Ishikawa, 1935). Determinations of pH of the intracellular environment by observation of change of color of dyes is obviously fraught with great sources of error. Nevertheless, it is a striking fact that colors corresponding to pH values ranging approximately from 3 to 5 have been observed by different investigators using different dyes and different phagocytic systems. It is worth noting, furthermore, that vital staining with litmus discloses that myriads of body cells, both those fixed in tissues and others appearing in exudates, can develop considerable granular acidity. Indeed, the polymorphonuclear elements and macrophages of a peritoneal exudate may become so laden with red litmus that the blue color of the fluid (alkaline) constituent is masked and the exudate appears a deep, turbid red (Rous, 1925).

If a burst of acidity occurs following engulfment of microorganisms, it is likely to have marked and probably deleterious effects on their physiological activity and viability, for it has often been reported that the cells of many microbial species die rapidly when exposed to acid reactions. Recent experiments have confirmed this fact and revealed furthermore that the nature of the acid present in the test solution is of great importance in determining the rate at which the microorganisms die (Table 13). *In vitro*, suspensions of many bacterial species are sterilized more rapidly at acid reactions in media containing lactic acid than in solutions containing hydrochloric acid, or phosphoric acid, or α-ketoglutaric acid, or dihydroxyacetone (Tables 14 and 15). Moreover, addition of keto acids, polycarboxylic acids or, dihy-

droxyacetone antagonizes to some extent the bactericidal effect of lactic acid (Tables 16 and 17; Dubos, 1953).

It is of obvious interest that lactic acid is so bactericidal at

TABLE 13

Survival of microorganisms at pH 4.0

Substance added (0.025 M)	Time (hr) required for 90-percent sterilization of bacterial suspension					
	Strep. (Group D)	*Coryne. creatinovorans*	*Klebsiella pneumoniae*	*Esch. coli*	*Salmonella typhimurium*	*Proteus vulgaris*
None	6+*	6+	6+	6+	6+	6+
Lactate	2	1	1	3	2	6+
Pyruvate	6+	6+	6+	3	6	6+
α-Ketoglutarate	6+	3	6+	6+	6+	6+
Glutamate	6+	3	6+	6+	6+	6+

Dubos, unpublished observations.

* + = longer than six hours.

TABLE 14

Effect of various metabolites on viability of staphylococci at acid reactions

Substance added to suspension of staphylococci (final concentration 0.01 M)	Number of viable staphylococci * after exposure at							
	pH 4.0 for (hr)				pH 5.5 for (hr)			
	1	2	3	5	2	5	24	48
Control	3×10^5	1×10^5	6×10^4	1×10^4	3×10^5			2×10^5
Acetic acid	2×10^5	3×10^4	0	0	1×10^5	1×10^4	0	0
Propionic acid	2×10^5	3×10^4	1×10^4	0	2×10^5	1×10^5	1×10^4	
Butyric acid	3×10^5	8×10^4	3×10^4	0	3×10^5	1×10^5	2×10^4	
Succinic acid	3×10^5	2×10^5	1×10^5	0	3×10^5	2×10^5	5×10^4	
Fumaric acid	3×10^5	2×10^5	1×10^5	0	3×10^5	3×10^5	3×10^4	
Citric acid	3×10^5	2×10^5	6×10^4	0	3×10^5	2×10^5	4×10^4	
Lactic acid	1×10^5	2×10^4	0	0	5×10^4	0	0	
Pyruvic acid	3×10^5	3×10^5	1×10^5	6×10^5	3×10^5	4×10^5	1×10^5	5×10^5
β-Hydroxybutyric acid	3×10^5	2×10^5	9×10^4	1×10^4	3×10^5	2×10^5	2×10^5	1×10^5
α-Ketoglutaric acid	3×10^5	3×10^5	2×10^5	1×10^5	3×10^5	3×10^5	4×10^5	7×10^5
Glutamic acid					3×10^5	2×10^5	2×10^4	9×10^3
Dihydroxyacetone	3×10^5	3×10^5	3×10^5	2×10^5	3×10^5	4×10^5	4×10^5	6×10^5

From Dubos, 1953*b*.

* Determined by duplicate plating on meat infusion–peptone agar.

TABLE **15**

Effect of various metabolites on viability of tubercle bacilli at acid reactions

Substance added to suspension of bacilli (final concentration 0.025 M)	Number of viable bacilli * after exposure at									
	pH 4.0 for (days)					pH 5.5 for (days)				
	1	2	3	6	8	3	5	7	10	14
Control	10^5	10^5	10^5	10^3	0	10^5	10^5	10^5	10^5	10^4
Acetic acid	10^4	10^3	0	0	0	10^2	0	0	0	0
Propionic acid	10^4	10^3	0	0	0	10^5	10^5	10^4	10^3	0
Butyric acid	10^4	10^3	0	0	0	10^5	10^5	10^4	10^4	0
Succinic acid	10^5	10^5	10^4	0	0	10^5	10^5	10^5	10^4	10^2
Fumaric acid	10^5	10^5	10^4	0	0	10^5	10^5	10^5	10^6	10^7
Citric acid	10^5	10^5	10^5	0	0	10^5	10^5	10^5	10^5	10^4
Lactic acid	10^4	0	0	0	0	0	0	0	0	0
Pyruvic acid	10^5	10^5	10^5	10^4	10^2	10^5	10^5	10^5	10^4	10^3
β-Hydroxybutyric acid	10^5	10^5	10^3	0	0	10^5	10^5	10^5	10^5	10^3
α-Ketoglutaric acid	10^5	10^5	10^5	10^2	0	10^5	10^5	10^6	10^8	10^8
Glutamic acid	10^5	10^5	10^5	0	0	10^5	10^5	10^6	10^8	10^8
Dihydroxyacetone	10^5	10^5	10^5	0	0	10^5	10^5	10^5		
Glycine	10^5	10^5	10^5	10^2	0	10^5	10^5	10^5	10^4	10^3
Alanine	10^5	10^5	10^4	0	0	10^5	10^5	10^4	10^3	10^2

From Dubos, 1953*b*.

* Determined by duplicate plating on albumin–asparagine agar. Calculated to the nearest exponential value of 10.

acid reaction in view of the fact that it is a product of the normal metabolism of phagocytic cells (Fleischmann and Kubowitz, 1927; Kempner and Peschel, 1930; McLeod and Rhoads, 1939; Stetson, 1951; McKinney *et al.*, 1953; Martin, McKinney, and Green 1953). It is tempting also to speculate on the fact that the presence of keto acids, dibasic acids, and dihydroxyacetone in the environment renders bacterial cultures more resistant to acid reactions (Dubos, 1953). These or related substances accumulate *in vivo* in certain abnormal metabolic states (uncontrolled diabetes, starvation, etc.) and one may wonder whether the intracellular environment of the phagocytes does not reflect the metabolic disturbances of the body as a whole. A decrease in the

TABLE **16**

Effect of various metabolites on toxicity of acetic and lactic acids for staphylococci at acid reactions

Substances added to suspension of staphylococci (final concentration 0.02 M)	Number of viable staphylococci * after exposure at								
	pH 4 for (hr)				pH 5.5 for (hr)				
	2	3	4	5	3	5	8	24	48
Acetic acid	1×10^5	4×10^4	0	0	3×10^4	0	0	0	0
Acetic acid + pyruvic acid	3×10^5	1×10^5	7×10^4	0	2×10^5	3×10^4	0	0	0
Acetic acid + α-ketoglutaric acid					3×10^5	2×10^5	9×10^4	4×10^4	0
Acetic acid + β-hydroxybutyric acid	8×10^4	3×10^4	0	0	2×10^5	8×10^4	2×10^4	0	0
Acetic acid + dihydroxyacetone	2×10^5	3×10^4	1×10^4		7×10^4	1×10^4	0	0	0
Lactic acid	2×10^4	0	0	0	4×10^4	0	0	0	0
Lactic acid + pyruvic acid	9×10^4	2×10^4	0	0	2×10^5	3×10^4	0	0	0
Lactic acid + α-ketoglutaric acid					3×10^5	1×10^5	7×10^4	2×10^4	1×10^4
Lactic acid + β-hydroxybutyric acid	2×10^4	5×10^3	0	0	3×10^5	2×10^5	5×10^4	0	0
Lactic acid + dihydroxyacetone	9×10^4	3×10^4	0	0	1×10^5	4×10^4	0	0	0

From Dubos, 1953*b*.

* Determined by duplicate plating on meat infusion–peptone agar.

amount of lactic acid produced as a result of phagocytosis, or the simultaneous presence of some other metabolite, might have a profound influence on the fate of engulfed microorganisms. Unfortunately, so little is known of the metabolism of phagocytic cells that one can do no more than speculate and quote a few isolated observations in support of the view that phagocytes too can exhibit metabolic abnormalities. It has been claimed for example, that the whole blood of diabetic patients, regardless of its sugar content, has a weaker bactericidal power than normal blood, and that this loss of activity is related more to acidosis than to any other biochemical disturbance accompanying diabetes; evidence has also been presented that while the phagocytes of rabbits made diabetic by alloxan treatment retain unimpaired the ability to engulf pneumococci, they lose some of their bac-

TABLE 17

Effect of various metabolites on toxicity of acetic and lactic acids for tubercle bacilli at acid reactions

Substances added to suspension of bacilli (final concentration 0.025 M)	Number of viable bacilli * after exposure at							
	pH 4 for (days)				pH 5.5 for (days)			
	1	2	3	6	3	5	7	10
Acetic acid	9×10^4	7×10^3	0	0	8×10^3	0	0	0
Acetic acid + pyruvic acid	2×10^5	5×10^4	0	0	9×10^3	3×10^3	0	0
Acetic acid + α-ketoglutaric acid	3×10^5	1×10^5	4×10^4	2×10^3	3×10^5	2×10^5	7×10^4	1×10^4
Acetic acid + β-hydroxybutyric acid	2×10^5	3×10^4	0	0	1×10^5	1×10^4	0	0
Acetic acid + glutamic acid					2×10^5	2×10^5	4×10^5	7×10^5
Lactic acid	4×10^4	0	0	0	2×10^4	0	0	0
Lactic acid + pyruvic acid	1×10^5	6×10^3	0	0	0	0	0	0
Lactic acid + α-ketoglutaric acid	3×10^5	4×10^4	0	0	2×10^5	1×10^5	0	0
Lactic acid + β-hydroxybutyric acid	8×10^4	4×10^3	0	0	5×10^3	2×10^3	0	0
Lactic acid + glutamic acid					1×10^5	2×10^5	4×10^5	9×10^5
Lactic acid + dihydroxyacetone					2×10^5	4×10^5	1×10^6	$3\times10_6$

From Dubos, 1953*b*.

* Determined by quadruplicate plating on asparagine–albumin agar.

tericidal power for these microorganisms (Cruickshank and Payne, 1949). On the other hand, there is some indication that the leucocytes of diabetics have an impaired glycolytic power which can be corrected back to normal by the addition of insulin, and that treatment of normal leucocytes with cortisone can bring about a decrease in lactic acid production (Martin, McKinney, and Green, 1953).

Any speculation concerning the possible role of the burst of acidity which follows phagocytosis as cause of death of the engulfed bacteria must take into account that many types of microorganisms (not only viruses and rickettsia but also bacteria, protozoa, and fungi) survive and multiply in the intracellular environment. In the absence of information one may ask whether

the resistant microorganisms possess some characteristic which makes them tolerant of the acid environment, or whether their presence within the cell fails to elicit a burst of acidity, or causes one of very short duration. It is possible also that the production of a phagocytic vacuole is not a constant phenomenon. Some types of microorganisms appear to remain free within the cytoplasm and they may be those that are capable of behaving as intracellular parasites. Tubercle bacilli belong to this group.

The hypothesis that intracellular acidity is one of the causes of the bactericidal effect of phagocytosis is at best a working hypothesis almost entirely devoid of experimental support. It has been presented here chiefly to bring to light our profound ignorance of one of the most important aspects of the pathogenesis of infectious diseases, namely, the lack of knowledge of the factors within the phagocytic cells which bring about the rapid death of certain bacteria, or which allow other microbial parasites to survive and proliferate in the intracellular environment.

ANTIMICROBIAL SUBSTANCES OF ANIMAL TISSUES AND BODY FLUIDS

It has long been known that one can separate from animal tissues and body fluids a variety of fractions possessing antimicrobial activity *in vitro* (reviewed by Nungester, 1951). The effects observed are often remarkably selective, a given preparation exhibiting high activity against one or a very few microbial types, and none against others under the same conditions. It is certain, therefore, that many different antimicrobial substances normally exist in animal tissues. The few that will be briefly considered in the following pages have been selected to illustrate (*a*) the wide range in their chemical nature, (*b*) the selectivity of their antimicrobial action, and (*c*) the fact that their activity is markedly influenced by factors of the environment, hence by the type of lesion in which they come into contact with microorganisms.

Lactenin. We shall begin our survey with this little-known

substance because it raises more enticingly than any other, perhaps, the possibility that certain normally occurring tissue products play a highly selective protective role against infection (reviewed by Wilson and Rosenblum, 1952).

Lactenin is a protein that has been separated from the whey fraction of nonpasteurized cow's milk. It is present in fairly low titers in colostrum reaching a relatively stable level 4 to 5 days *postpartum* and persisting thereafter in the milk. It possesses a high bactericidal power for hemolytic streptococci of group A, but as far as is known is much less active against other microorganisms, even against other streptococci. It is inactivated when converted to the reduced state, for example in the presence of –SH groups, so that its antibacterial activity manifests itself only under aerobic conditions.

There is little doubt that the presence of lactenin in unheated milk helps in minimizing the spread of milk-borne group A streptococci among men, but there is as yet no convincing evidence that it plays any part in protecting animals against infection under natural conditions. Yet several facts can be construed to attribute to lactenin a role of this sort, namely, its abundance *postpartum*, that is, at a time when protection of the milk is essential both to the cow and to the calf; the selectivity of its antibacterial action for hemolytic streptococci, the most virulent agents of bovine mastitis; the fact that it is active in the oxidized state, that is, under aerobic conditions such as prevail in the udder.

Complement. It is well known that the ability of complement to cause the lysis of susceptible cells in association with specific antibody depends upon the participation of four different serum constituents. These must be present in the right proportions, the component which is deficient in amount in a given system becoming the rate-limiting factor. Although the ability of complement to cause cytolysis in the presence of specific antibody is always emphasized, it is certain that the complement-antibody complex can kill many types of cells in addition to those which

it can lyze. All species of Gram-negative bacilli seem susceptible to this killing effect, but apparently not Gram-positive bacteria. There is some evidence that certain protozoa and viruses are also susceptible.

The mechanism of the cytotoxic and lytic effects is far from clear, but studies of immune hemolysis suggest that the phenomenon involves several independent steps. At least one of the reactions of the early phases requires calcium, and another magnesium; this is followed by a terminal phase which can occur at 0° C and results in the liberation of hemoglobin (Mayer *et al.*, 1952, 1953; Levine *et al.*, 1953). Along with the fixation of complement at the red-cell surface by antibody and divalent cations, there seems to occur an enzymatic reaction which produces damage to the ultra-structure of the cell membrane.

Some peculiar facts recently brought to light suggest that the relation between cell and complement may be extremely indirect. If a heterologous antigenic substance is adsorbed on a bacterial surface, an antibody specific for this antigen can sensitize the microorganisms to the killing effect of complement (Adler, 1952). Similarly, chicken erythrocytes sensitized with PR8 influenza virus are lyzed by complement in the presence of specific virus antibody (Cox, Zerschling, and Meloy, 1953). Since the antigen involved in hemolysis need not be a constituent of the erythrocyte, it is unlikely that the complement acts directly on the cellular structure. Instead, it seems that the reaction which occurs between antigen and antibody in the presence of complement and in the immediate vicinity of the cell constitutes the mechanism responsible for the toxic effect. In this regard, it is worth mentioning that antigen-antibody reactions occurring in the presence of complement can bring about the activation of the serum proteolytic system (see page 90), and one may wonder whether the release of a cytotoxic factor may not be the indirect consequence of the activation of serum proteases.

Although the antimicrobial effect of complement is easy to demonstrate *in vitro*, and extends to many pathogenic species,

there is no convincing evidence that it operates *in vivo* as a protective mechanism against infection. It is of interest to mention in this respect reports suggesting that complement does not exist in the free state in circulating blood and is liberated only as a result of trauma, for example, caused by the withdrawing of blood under ordinary conditions (Wollmann, 1913; Fuchs, 1930, 1933). It appears possible, therefore, that complement is liberated and can exert locally its antimicrobial effect precisely in areas where microbial multiplication is sufficient to produce necrosis (Maaloe, 1948).

Enzymes. Despite many reports to the contrary, there is no convincing evidence that proteolytic enzymes can attack living microbial agents (Salton, 1953). It is true that many tissue extracts rich in proteolytic enzymes — pancreatic extracts in particular — can inactivate bacteria and even viruses, but these correct experimental findings have led to erroneous interpretations (Pirie, 1935). On the one hand, tissue extracts contain a great variety of substances possessing antimicrobial activity; on the other hand, their enzymes can bring about the production from tissue substrates of split products and derivatives which are responsible for the antimicrobial effect (see following pages of this chapter). Lipases in particular will liberate toxic fatty acids; and even trypsin, acting on tissue constituents, can separate from protein complexes toxic lipids bound to them in an inactive form. However, since cathepsin-like proteases of plants (ficin and bromelin) are known to attack living mammalian cells and helminths, it is also possible that special tissue cathepsins can destroy certain types of living microbial agents. But this has not yet been proved.

It might be mentioned here that certain proteolytic enzymes can hydrolyze protein components of the surface of living hemolytic streptococci of group A and of anthrax bacilli (Elliott, 1945, 1954; Stamp, 1953; Heckly and Goldwasser, 1949). Although proteolytic destruction of the surface antigens does not decrease the viability of these organisms, it deprives them of a constituent essential for their pathogenic behavior.

Only one enzyme has been shown so far to possess bactericidal activity, namely, lysozyme, which hydrolyzes an acetylamino polysaccharide constituent of bacterial membranes (reviewed by Dubos, 1945). It is a basic protein of small molecular weight which rapidly lyzes the living cells of a few bacterial species; in addition, there are many more species which are killed by the enzyme, although not lyzed. Lysozyme occurs in many types of animal tissues and body fluids. Of particular interest is the fact that it is present in high concentrations in granulocytes and can be released in an active form into the environmental fluid as the result of any injury to these cells, even minor (Hiatt *et al.*, 1952; Kerby, 1952). In consequence lysozyme occurs in large amounts where inflammatory cells accumulate (Moeller, Marshall, and Kirsner, 1951) and may play a role in retarding or stopping microbial proliferation in these areas. Its antibacterial activity is inhibited by certain polysaccharide acids, with which the enzyme probably forms insoluble inactive complexes; in contrast, it is enhanced by histone (Kaiser, 1953; Kerby and Eadie, 1953). The presence of various components and breakdown products of tissues may be of importance, therefore, in determining the effectiveness of lysozyme as an antimicrobial agent in inflammatory areas.

Basic peptides. From several types of animal tissues there have been separated at least two different basic peptides which can inhibit the growth *in vitro* of several bacterial species. One, with a high lysine content, was detected through its activity against *Bac. anthracis* but is also active against *Bac. subtilis*, *Bac. megatherium*, *Staphylococcus aureus*, *Streptococcus hemolyticus*, *Escherichia coli*, and certain viruses (Watson *et al.*, 1947; Watson and Bloom, 1952). The other is characterized by a high arginine content and is especially active against tubercle bacilli (Tables 18–21; Dubos and Hirsch, 1954; Hirsch, 1954).

The antibacterial activity of both peptides decreases markedly with increasing acidity. That of the lysine peptide is also inhibited by nucleic acids as well as by other negatively charged

large molecules of common occurrence in animal tissues. Curiously enough, the arginine compound is not affected by these substances, but is partly neutralized by addition of sulfate or organic sulfur compounds to the culture media (Tables 18–21).

TABLE **18**

The antimycobacterial activity of various basic peptides

Final concentration of basic peptide (μg/ml)	Growth of tubercle bacilli (BCG-Phipps) in the presence of basic peptide added to the medium				
	Thymus peptide	Spleen polylysine	Pituitary corticotropin (ACTH)	Posterior pituitary pressor	Posterior pituitary oxytocic
100	0	0	0	+++	++++
30	0	+	+	++++	++++
10	+	+	+	++++	++++
3	+	++++	++	++++	++++
1	++	++++	++++	++++	++++
0.3	++++	++++	++++	++++	++++

From Hirsch, 1954.

TABLE **19**

The influence of the concentration of $MgSO_4$ in the medium on the antimycobacterial activity of thymus peptide

Final concentration of thymus peptide (μg/ml)	Growth of tubercle bacilli (BCG-Phipps) in a medium containing a final concentration of $MgSO_4 \cdot 7H_2O$ of			
	0.1 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml
100	0	0	0	+
30	0	0	+	+++
10	+	+	++	++++
3	+	+	++++	++++
1	+	+	++++	++++
0.3	+	++++	++++	++++
0.1	++	++++	++++	++++
0.03	++++	++++	++++	++++
None	++++	++++	++++	++++

From Hirsch, 1954.

TABLE **20**

The influence of various magnesium salts in the medium on the antimycobacterial activity of thymus peptide

Final concentration of thymus peptide (μg/ml)	Effect on the growth of tubercle bacilli of the addition to the medium of 50 μg/ml				
	$MgSO_4 \cdot 7H_2O$	$MgCl_2 \cdot 6H_2O$	$Mg(NO_3)_2 \cdot 6H_2O$	Mg citrate	None
10	++++	+	+	+	+
None	++++	++++	++++	++++	++++

From Hirsch, 1954.

TABLE **21**

Antagonism of the antimycobacterial activity of thymus peptide by the addition of sodium sulfate to the medium

Final concentration of thymus peptide (μg/ml)	Growth of tubercle bacilli (BCG-Phipps) in the presence of Na_2SO_4 added to the medium to give a final concentration (μg/ml) of			
	None	30	100	300
50	0	0	0	0
25	0	+	+	+
12.5	+	+	+	+++
6.25	+	+	++	++++
3.125	+	++	++++	++++
1.56	++	+++	++++	++++
None	++++	++++	++++	++++

From Hirsch, 1954.

There is no proof that these basic peptides do exert a protective action against bacteria *in vivo*, but it has been recently shown that polylysine (synthetic) is active against certain virus infections in the chick embryo (Green and Stahmann, 1953; Green, Stahmann, and Rasmussen, 1953). It seems possible that under certain conditions tissue damage might result in the release of basic peptides from more complex organic structures in the immediate environment of the necrotic tissue where the infectious

agents are located, and thus permit antibacterial action to take place without interference from other tissue constituents.

Spermine, spermidine, and spermine oxidase. The search for antimycobacterial substances in animal tissues has led to the recognition that the polyamines spermine and spermidine can cause the death of mammalian tubercle bacilli under certain conditions (Dubos, 1951; Hirsch and Dubos, 1952; Hirsch, 1953). Interestingly enough, these substances are devoid of antimycobacterial activity in synthetic culture media. But they develop bactericidal power, even in dilutions as high as 1/300,000, after they have been acted upon by an enzyme which has been called spermine oxidase (Hirsch, 1952, 1953). It is the product — as yet unidentified — of the enzymatic oxidative deamination of spermine and spermidine which is the true bactericidal agent (Tables 22–24).

Spermine and spermidine occur in many animal and human tissues in concentrations larger than those sufficient to kill tu-

TABLE 22

The influence of the concentration and purity of the bovine albumin in the medium on the antimycobacterial activity of spermine

Bovine albumin preparation added to the medium	Final concentration (percent)	Inhibition of growth of tubercle bacilli * Molar concentration of spermine				
		7×10^{-5}	3.5×10^{-5}	1.75×10^{-5}	8.5×10^{-6}	None
Plasma fraction V	0.3	++++	++++	+	0	0
	0.1	++++	++++	++++	0	0
	0.03	++++	++++	++++	0	0
	0.01	++++	++++	++++	+	0
	0.003	++++	+	0	0	0
	None	0	0	0	0	0
Crystalline albumin	0.3	0	0	0	0	0
	0.1	0	0	0	0	0
	0.03	0	0	0	0	0
	0.01	0	0	0	0	0
	0.003	0	0	0	0	0
	None	0	0	0	0	0

From Hirsch, 1953*a*, *b*, *c*.

* ++++ = complete inhibition of growth; 0 = no inhibition of growth.

bercle bacilli *in vitro*. But since these polyamines are inactive *per se*, the presence of spermine oxidase and the proper environmental conditions for oxidative deamination are the limiting

TABLE **23**

The activation of spermine by serum from various animals

Whole serum added to the medium	Final concentration (percent)	Inhibition of growth of tubercle bacilli		Whole serum added to the medium	Final concentration (percent)	Inhibition of growth of tubercle bacilli	
		No spermine	5×10^{-5} M spermine			No spermine	5×10^{-5} M spermine
Beef	10	0	++++	Rabbit	10	0	0
	1	0	++++		0.1	0	0
	0.1	0	++++		0.001	0	0
	0.01	0	++++				
	0.001	0	++++	Guinea pig	10	0	0
	0.0001	0	0		0.1	0	0
					0.001	0	0
Sheep	10	0	++++				
	1	0	++++	Horse	10	0	0
	0.1	0	++++		0.1	0	0
	0.01	0	++++		0.001	0	0
	0.001	0	++++				
	0.001	0	0	Pig	10	0	0
					0.1	0	0
Man	10	0	0		0.001	0	0
	0.1	0	0				
	0.001	0	0	No serum added	—	0	0

From Hirsch, 1953*a*, *b*, *c*.

TABLE **24**

The spermine activator content of aqueous extracts of guinea-pig and rabbit organs

Organ extract added to the medium	Final concentration (percent)	Inhibition of growth of tubercle bacilli		Organ extract added to the medium	Final concentration (percent)	Inhibition of growth of tubercle bacilli	
		No spermine	5×10^{-5} M spermine			No spermine	5×10^{-5} M spermine
Guinea-pig kidney	10	0	++++	Rabbit kidney	10	0	0
	2	0	++++		2	0	0
	0.4	0	0		0.4	0	0
Guinea-pig heart	10	0	++	Rabbit heart	10	0	0
	2	0	0		2	0	0
	0.4	0	0		0.4	0	0

From Hirsch, 1953*a*, *b*, *c*.

factors of antibacterial activity. One example will be given to illustrate the possible significance of these facts in pathogenesis. In guinea pig tuberculosis, the kidneys usually remain free of disease, even when virulent bacilli are injected directly into renal tissue. In contrast, the kidneys of the rabbit are extremely susceptible to tuberculous infection. The fact that the enzyme spermine oxidase is present in guinea pig kidney, but not in rabbit kidney, suggests that the spermine–spermine oxidase system might play a role in natural resistance to tuberculous infection. It must be emphasized, however, that too little is known of the distribution of the enzyme, and of the conditions under which it functions, to justify any conclusion at this time.

Heme compounds. It has long been known that several types of heme compounds are highly toxic for a variety of microorganisms, particularly certain Gram-positive bacilli and cocci (reviewed in van Heyningen, 1951; Ivanovics and Koczka, 1952). The iron-free porphyrin derivatives are inactive in this respect, and indeed many of them act as growth factors used for the biosynthesis of the heme respiratory pigments by some microbial species. It is likely that the antibacterial effect of hemes depends upon their ability to act as antimetabolites by competing with porphyrins essential for metabolism and growth (Table 25; Granick and Gilder, 1946; Lwoff, 1948).

Free heme compounds probably do not accumulate in high concentrations in tissues under ordinary circumstances. But they are active in such high dilutions against certain microorganisms that their antibacterial effect may manifest itself in localized areas. For example, it was found in unpublished experiments that heme concentrations sufficient to kill staphylococci can be released by exposing blood diluted 10,000-fold for a few minutes at pH 4.5–5.0 (Table 26). This fact is of some interest in view of the recent finding that in the presence of specific antibody microorganisms can be adsorbed on erythrocytes and phagocytized with them (Nelson, 1953). If the pH within the phagocytic vacuole is really as low as appears from observations with pH

TABLE **25**

Competition between protoporphyrin and hematoporphyrin in cultures of *H. influenzae*

Additives to medium (μg/ml)		Metabolic Effect	
Protoporphyrin	Hematoporphyrin	Growth	Nitrate reduction
0.00		−	−
.01		±	±
.03		++++	++++
.1		++++	++++
0.03	0.10	+++	+++
.03	.20	++	++
.03	.40	±	−
.03	.60	−	−
0.10	0.20	+++	++
.10	.60	++	+
.10	1.00	±	−

Condensed from Granick and Gilder, 1946.

TABLE **26**

Bactericidal effect of heme on *St. aureus*

Substance added to broth	Percent	Staphylococci (percent) surviving 2 hr at pH:			
		4.5	5.0	6.0	7.0
None		90	100	100	100
Blood	0.01	10	100	100	100
Hemoglobin	.001	5	100	100	100
Heme	.0003	0	0	0	0

Dubos, unpublished observations.

indicators (see page 37), enough heme might be released locally to have some antibacterial effect. Relevant to this problem is also the fact that pus cells contain large amounts of verdoperoxidase, the heme-protein complex responsible for the color of green pus (Agner, 1941). Although the heme of verdoperoxidase is not released from its protein complex as readily as that of hemoglobin,

the enzymatic processes during autolytic necrosis of leucocytes may be capable of separating the iron porphyrin from its protein moiety. Thus heme compounds might be set free by the breakdown of erythrocytes and leucocytes in inflammatory areas.

Lipids, organic acids, and CO_2. The selective antibacterial activity of certain lipids and bile acids *in vitro* is often made use of in the preparation of diagnostic culture media — for example, oleic acid inhibits Gram-positive cocci in media designed for the isolation of *Hemophilus influenzae*, and bile acids facilitate the isolation of enteric pathogens.

Observations *in vivo* are less convincing but sufficiently suggestive to warrant discussion. It seems likely that the fatty acids of the skin are responsible, in part at least, for the self-sterilizing power of that organ. In pneumococcus pneumonia, the large amounts of long-chain fatty acids which accumulate in the consolidated lung may contribute to the early disappearance of pneumococci from the center of the lesion (Lamar, 1911; Lord, 1919; Kelley *et al.*, 1938); this is rendered more likely by the fact that the toxic action of fatty acids is magnified at the acid reactions prevailing in the pneumonic lesion. Long-chain fatty acids are toxic for a number of other microbial species, streptococci, staphylococci, tubercle bacilli, and, as already pointed out, for certain viruses. In tissues undergoing necrosis, autolytic enzymes may cause the release and local accumulation of fatty acids in concentrations sufficient to exert some antibacterial effect; free fatty acids are certainly present in high concentrations in the caseous areas of tuberculosis (reviewed in Wells and Long, 1932).

We have seen on the other hand that inflammatory sites are usually more acid than the normal extracellular environment — in large part through the activities of inflammatory cells which have a predominantly glycolytic mechanism and produce large amounts of lactic acid (see pages 15 and 37). Recent experiments with tubercle bacilli, staphylococci, and Friedlander bacilli indicate that, at reactions which are presumed to prevail commonly within inflammatory zones (approximately pH 6.5), lactic acid

can exert marked bacteriostatic and even bactericidal activity. This antibacterial effect is markedly enhanced in the presence of CO_2 and under anaerobic conditions (Dubos, 1952, 1953; Weiss and Dubos, unpublished observations), a finding rendered more significant by the fact that the tension of oxygen is very low, and that of CO_2 abnormally high, in the very areas where lactic acid accumulates (Tables 27–30).

TABLE **27**

Effect of sodium lactate on survival of tubercle bacilli (BCG-P) under anaerobic conditions at pH 6.8

Sodium lactate added (percent)	Conditions of incubation	Number of viable bacilli present after following intervals of time (weeks) at 37.5° C *			
		0	1	3	4
0.4	Anaerobic	10^8	10^6	10^3	0
.2	Anaerobic	10^8	10^7	10^4	0
.1	Anaerobic	10^8	10^7	10^5	10^2
.05	Anaerobic	10^8	10^7	10^5	10^6
0	Anaerobic	10^8	10^7	10^6	10^6
0.4	Aerobic	10^8	10^9	10^8	10^8
	Aerobic	10^8	10^8	10^7	10^7

From Dubos, 1953*a*.

* The figures give the numbers of colonies (calculated to the nearest whole exponential value of 10) recovered per milliliter of culture.

The concentration of carbon dioxide in the gaseous and aqueous environment can also have a direct effect on the fate and properties of microorganisms *in vivo*. Tubercle bacilli grow poorly or not at all in atmospheres rich in CO_2. As stagnant tissue may contain up to 6.5 percent carbon dioxide, it seems not unlikely that some of the therapeutic results of pneumothorax may be due to this inhibitory effect (Davies, 1940). In contrast, the growth of some bacterial species is stimulated as CO_2 tension increases, a fact first shown with *Brucella* but also true of other species.

Needless to say, the biochemical environment created by inflammation differs from the normal "*milieu intérieur*" in a man-

TABLE **28**

Effect of Na lactate (0.2 percent) and alanine (0.2 percent) on the viability of various strains of tubercle bacilli under anaerobic conditions at pH 6.8

Culture	Substance added	Number of viable tubercle bacilli present after the following intervals of time (weeks) at 37.5° C							
		1		2		3		4	
		Aerobic	An-aerobic	Aerobic	An-aerobic	Aerobic	An-aerobic	Aerobic	An-aerobic
MV	0	10^8 *	10^9	10^8	10^8	10^8	10^8	10^8	10^7
MV	Lactate	10^9	10^8	—	10^6	—	10^3	10^8	10^1
MV	Alanine	10^9	10^8	—	10^8	10^8	10^6	—	10^3
H37Rv	0	10^8	10^8	—	10^8	—	10^7	10^8	10^6
H37Rv	Lactate	10^9	10^8	—	10^4	—	10^4	10^8	10^2
H37Rv	Alanine	—	—	—	—	—	—	—	—
R1Rv	0	10^8	10^8	—	10^8	—	10	—	10^6
R1Rv	Lactate	10^9	10^8	—	10^7	—	10^5	10^8	0
R1Rv	Alanine	10^9	10^8	—	10^7	—	10^6	10^8	10^3
BCG—P	0	10^8	10^8	—	10^8	—	10^7	10^8	10^8
BCG—P	Lactate	10^9	10^8	—	10^5	—	10^2	10^8	0
BCG—P	Alanine	10^9	10^8	—	10^6	—	10^6	10^8	10^2
BCG-T	O	10^8	10^8	—	10^5	—	0	10^8	0
BCG-T	Lactate	10^8	10^8	—	10^4	—	0	10^5	0
BCG-T	Alanine	—	—	—	—	—	—	—	—

From Dubos, 1953*a*.

—, not done.

* The figures give the numbers of colonies (calculated to the nearest whole exponential value of 10) recovered per milliliter of culture.

ner much more complex than indicated in the preceding pages. Changes in blood flow and in permeability of the vascular bed alter considerably the supply and removal of various substances. Moreover, it is likely that many types of pathological conditions bring about profound changes in the kind and amount of inorganic and organic ions which accumulate in infected areas. In addition to lactic acid and CO_2, other normal and abnormal metabolites are produced and released by fixed and wandering cells. It is worth mentioning in this respect that certain dicarboxylic and keto acids greatly facilitate the growth of many bacterial species and can even replace CO_2 as growth factor (Lwoff and Monod, 1947; Ajl and Werkman, 1949; reviewed by Werkman, 1951, p. 422; Tables 31 and 32). They also antagonize the antibacterial

TABLE 29

Effect of gaseous environment on the fate of tubercle bacilli

Gaseous environment		Strain of tubercle bacilli	Number of viable tubercle bacilli present after following periods of time (weeks) at 37.5° C*				
Oxygen	CO_2		0	1	2	3	4
−	+	BCG–P	10^8	10^5	10^3	0	0
−	−	BCG–P	10^8	10^7	10^4	10^3	0
+	−	BCG–P	10^8	10^8	10^8	10^8	10^8
+	+	BCG–P	10^8	10^8	10^8	10^8	10^8
−	+	RlRv	10^9	10^3	10^1	0	0
−	−	R1Rv	10^9	10^7	10^4	0	10^2
+	+	R1Rv	10^9	10^8	10^8	10^7	10^7

Condensed from Dubos, 1953*a*.

* The figures give the numbers of colonies (calculated to the nearest whole exponential value of 10) recovered per milliliter of culture.

TABLE 30

Effect of CO_2 on the fate of staphylococci under anaerobic conditions

CO_2	Colonies * obtained from medium at pH	
	5.2	6.8
+	72	75
−	0	3

Condensed from Weiss and Dubos, 1954, unpublished.

* Number of colonies, to be multiplied by 2×10^5, recovered per milliliter of bacterial suspension in nutrient broth incubated for 3 days at 37° C in anaerobic jar with or without CO_2.

effect of many injurious agents, particularly of lactic acid under aerobic and anaerobic conditions, as can also other ketone bodies, and certain polycarboxytic acids and amino acids (Dubos, 1953; Heinmets *et al.*, 1954; Weiss and Dubos, unpublished observations, see Tables 16 and 17).

It is not yet known to what extent the effects observed *in vitro* have a bearing on the events which occur *in vivo*. Nevertheless,

TABLE 31

The effect of organic acids on the growth of *E. coli* in the absence of CO_2

Substance added	Percent	Growth after incubation for (hr)	
		18	40
0		−	−
Acetate	0.01	−	−
Propionate	.01	−	−
Butyrate	.01	−	−
Succinate	.002	+	+
Glutarate	.001	+	+
Aspartate	.001	+	+
Oxalate		−	+
CO_2		+	+

Condensed from Lwoff and Monod, 1947.

TABLE 32

Replacement of carbon dioxide by α-ketoglutaric acid in the growth of *A. aerogenes*

Additions to basal medium *	Concentration	Growth in the absence of CO_2
No additions	—	5
Ammonium sulfate	0.4%	10–15
Ammonium sulfate α-Ketoglutaric acid	0.4% 0.5 mM	325
DL-Alanine	4 mM	50
DL-Alanine α-Ketoglutaric acid	4 mM 0.5 mM	425

Condensed from Ajl and Werkman, 1949.

* The basal medium contained 0.8 percent KH_2PO_4, 0.8 percent glucose, 10 percent tap water (for inorganic ions), made up to 100 ml with distilled water. To this were added the various compounds listed plus 1 ml of a 24-hr culture of *A. aerogenes*. It was aerated with CO_2-free water for 18 hr at 30° C; growth was expressed in terms of turbidimetric readings on the photoelectric colorimeter, using 660-mμ filter.

one cannot doubt that the availability of oxygen, the tension of CO_2, the reaction of the body fluids, and the presence and concentration of various organic acids and other metabolites, are all factors which give to inflammatory areas a peculiar biochemical character and influence thereby the survival and multiplication of infectious agents *in vivo*.

QUALITATIVE CHANGES IN MICROBIAL POPULATIONS

The physicochemical factors of the *in vivo* environment influence the course of the infectious process not only by determining the numbers of microorganisms which survive and multiply, but also by modifying their qualitative characteristics. This is not surprising in view of the fact, so extensively studied *in vitro*, that microorganisms exhibit an extraordinary plasticity which manifests itself in the form of hereditary population changes resulting from the selection of mutants, and of nontransmissible phenotypic modifications which are a more direct expression of their response to the environment. Some of these changes — either phenotypic or hereditary — happen to enhance the pathogenic propensities of the infectious agent as if they were successful adaptive attempts on its part to overcome the defense mechanisms of the infected host. Other changes, on the contrary, decrease pathogenicty and therefore appear to be the result of a successful defensive reaction of the host. In reality, however, the infectious agents can undergo *in vivo* all sorts of qualitative changes which may affect the course of infection in different and often opposite ways. Only a few have been recognized so far, chiefly those having to do with characters detectable by immunological tests.

It has been known for several decades that changes in antigenic structure of bacteria (as well as protozoa and viruses) can be brought about by cultivation in media containing homologous antibody (reviewed by Luria, 1947; Braun, 1947); more recently techniques have been developed to induce at will *in vitro* hereditary changes in the composition and properties of bacterial cells

(and perhaps also of viruses) by exposing them under the proper conditions to desoxyribonucleic acid fractions of the organism possessing the desired attribute (reviewed by Austrian, 1952, and Hotchkiss, 1954). Thus, strains of bacteria may be made to acquire new serological specificities, enzymatic properties, or degrees of resistance to drugs. These phenomena, which may also occur *in vivo*, have been well described in reviews or textbooks, and cannot be discussed here.

It is a striking fact that some of the properties of certain infectious agents are related to the age of the host in which they are found. For example, *Hemophilus influenzae* is found almost exclusively in the R form in adults, whereas its S forms are a common cause of acute disease in children. In a few cases, some knowledge is available of the metabolic factors which condition the selection of mutants, and thus bring about hereditary changes in the microbial population. *Brucella* grown in media in which alanine is allowed to accumulate tend to change from the S to the R colonial type. This change requires the presence of Mn^{++} ions and is inhibited by the presence in the medium of chelating agents which bind this cation* (Tables 33–35). It is also under the control of some serum constituents, the concentration of which

TABLE **33**

Population changes in "S" *Brucella suis* cultures in synthetic asparagine medium

Amino acid in medium	M and R mutants † found after following days					Amino acid accumulating in culture
	5	8	12	16	20	
l-Asparagine	0	0	42% M	52% M	41% M	Valine
d-Asparagine	16% R	55% R	84% R	90% R	97% R	Alanine

From Braun, W., 1952.
† M = mucoid; R = rough.

* It has been recently shown that iron, and not manganese, is probably the cation responsible for the effects on the dynamics of bacterial population observed in these experiments (Waring *et al.*, 1953).

TABLE **34**

Population changes in "S" *Brucella abortus* cultures in synthetic asparagine medium.

Day of Growth	Non-"S" mutants (percent) appearing in synthetic medium containing additives:			
	Nil	Filtrate of old culture	Alanine (d,1-)	
			100 μg/ml	200 μg/ml
2	0	0	0	0
4	0	0	1	2
6	0	1	6	19
8	0	2	14	36
11	2	64	43	83
14	6	91	57	86
19	76	91		

From Braun, W., 1952.

TABLE **35**

Inhibition of variation in *Brucella abortus* ("S") by chelating agents with reversal by Mn^{++}

Chelating agent	Metal *	Nonsmooth variants † (percent)
None	Nil	35
Ethylenediamine tetra acetate, 1.8×10^{-4} M	Nil	0
	Mn^{++}, 4×10^{-4} M	47
	Mg^{++}, 1×10^{-2} M	0
	Na^{+}, 3×10^{-3} M	0
8-hydroxyquinoline, 2×10^{-5} M	Nil	0
	Mn^{++}, 4×10^{-3} M	15
Na citrate, 8×10^{-3} M	Nil	0
	Mn^{++}, 4×10^{-3} M	15

From Cole, 1952, unpublished (in Braun, W., 1952).

* It has been recently shown that iron, and not manganese, is probably the cation responsible for the effects on the dynamics of bacterial population observed in these experiments (Waring, Elberg, Schneider, and Green, 1953).

varies from one species of animal to another, and with the state of disease or of immunity (Braun, W., 1952). As the S *vs.* R colonial characteristics in *Brucella* are of significance for pathogenicity, we have here an inkling of the manner in which the biochemical environment can affect the course of the infectious process by modifying the microbial population through hereditary changes in its components.

We shall now consider a few examples illustrating the influence of the physicochemical environment on the nontransmissible manifestations of pathogenic potentialities. Probably the most striking known case is the influence of iron on the production of diphtheria toxin. The presence of this metal in the medium is essential for the multiplication of diphtheria bacilli, but an excess of it — while not inimical to bacterial growth — decreases or can even prevent toxin production (Pappenheimer, 1947). The metabolic basis of the relation of iron concentration to yield of toxin is discussed in a later chapter (see page 70). Suffice it to mention here that an inhibition of toxin production by iron has been observed *in vitro* also in the case of tetanus toxin (Mueller and Miller, 1945) and particularly of Shiga neurotoxin (Dubos and Geiger, 1946; Van Heyningen and Gladstone, 1953). It is not unlikely that these phenomena are of significance in disease as the amounts of free iron found to have inhibitory effects on toxin production are of the order of those that may occur *in vivo* (Mueller, 1941).

There are many other examples of the influence of the biochemical environment on the production of toxin; thus, the lethal toxin of hemolytic staphylococci is produced most abundantly during growth under high CO_2 tension (Gladstone, 1938; Duthie and Lorenz, 1952); the O hemolysin of streptococci reaches high concentrations only in media containing certain nucleotides (Bernheimer, 1948). The edema-producing factor of *Bac. anthracis*, which had long been assumed to occur only *in vivo*, has recently been obtained *in vitro* in media containing serum constituents not required for abundant bacterial growth (Gladstone, 1948;

Heckly and Goldwasser, 1949). Recent experiments have revealed furthermore that anthrax bacilli growing in the tissues of infected guinea pigs differ in still other respects — as yet ill defined — from bacilli grown in vitro (Smith, Keppie, and Stanley, 1953).

Mention should also be made of the fact that the production of microbial enzymes is markedly dependent upon the composition of the culture medium, particularly in the case of the so-called "adaptive enzymes" which are produced as a specific response to the presence of the homologous substrate (or a related substance) in the medium (reviewed by Monod, 1947). So far, only one enzyme of interest in pathogenesis has been shown to be adaptive in nature, namely, hyaluronidase (spreading factor) (Rogers, 1946), but it is certain that many other enzymes of adaptive type can be produced by pathogenic microorganisms.

Although we have considered separately the hereditary and the phenotypic changes imposed upon microorganisms by the environment, it is obvious that both take place simultaneously *in vivo*. We shall now briefly consider two examples illustrating the complex interplay between hereditary characters of microorganisms and the physicochemical conditions prevailing in the tissues where the infectious process takes place.

As human patients recover from infection with streptococci of group A, the organisms isolated from their nasopharynx are found to contain smaller and smaller amounts of the type specific "M" protein, a surface antigen known to be an essential factor of virulence in this microbial species (Rothbard and Watson, 1948). This change is commonly believed to be the result of the growth of the streptococci in the presence of the homologous "M" antibody elicited as a response to infection. It is known also that "M" protein is readily destroyed by several types of proteolytic enzymes, particularly by a cathepsin produced by the streptococci themselves.

As a rule streptococci which produce large amounts of cathepsin yield small amounts of the "M" protein, indicating that the

quantitative manifestation of the "M" character of a culture is determined not only by the ability of this culture to produce the protein, but also by the readiness with which this substance is destroyed by enzymatic action under the conditions of growth. In turn, the intensity of enzymatic destruction is an expression both of the hereditary characteristics which control the production of enzyme and of the factors of the environment which control its activity. As the streptococcus cathepsin is most active at low pH and only when present in a reduced form (presence of a –SH radical) (Elliott, 1945, 1954; Stamp, 1953), destruction of the "M" protein is more likely to occur under somewhat acidic and reducing conditions. All these facts are of importance in pathogenesis, since proteolytic destruction of the "M" protein results in a decrease of resistance of the streptococcus to phagocytosis, with consequent loss of virulence. A similar situation seems to obtain in the case of anthrax (Gladstone, 1948; Heckly and Goldwasser, 1949). One of the immunizing surface antigens of the virulent agents of this disease is a complex peptide which can be broken down by a variety of proteolytic enzymes and in particular by a protease of *Bac. anthracis* itself.

The pneumococcus provides another example where both the transmissible characteristics of the organism and the nature of the environment play a part in affecting a surface component of the microbial cell which is of importance in the infectious process. As is well known virulent pneumococci differ chemically and serologically in the nature of the polysaccharides which constitute their capsules. Strains differ not only qualitatively with reference to the chemical nature of the capsular polysaccharide, but quantitatively in the amount of capsular material produced (MacLeod and Krauss, 1953). For each strain, the chemical nature and the quantity of capsular material are controlled by hereditary characteristics which therefore constitute one of the determinants of virulence. But it is known, on the other hand, that many factors of the environment affect both the production of the capsular polysaccharide and its accumulation around the cell. Thus, the

polysaccharide of type III pneumococci is most readily formed and least soluble from pH 6.3 to pH 6.7 and therefore more likely to persist in the form of a capsule around the bacterial cell at this acidic reaction (Bernheimer, 1953).

For lack of precise knowledge, it is not possible to elaborate further on the fact that the physicochemical characteristics of the *in vivo* environment are of paramount importance in determining the extent to which are expressed the pathogenic potentialities of a given microorganism. Suffice it to suggest that many toxic manifestations of infectious diseases for which there is at present no adequate explanation may turn out to be caused by substances — either poisons or enzymes — which have not yet been recognized *in vitro* because they occur only as a response of the infectious agent to the presence of some factor peculiar to the *in vivo* environment.

CHAPTER 3

Biochemical disturbances produced by infection

TOXEMIA AND TISSUE DESTRUCTION

In his book, *A new theory of consumption, more especially of a phthisis or consumption of the lungs*, published in 1722, Benjamin Marten suggested that the "Essential Cause" of Consumption might be "Certain Species of *Animalculae* . . . that by their peculiar Shape or disagreeable Parts are inimicable to our Nature," and cause "a wearing away or consuming of all the muscular or fleshy Parts of the Body." Thus Marten was probably the first to express, long before the demonstration of the germ theory of disease, the possibility that microorganisms can have toxic and destructive effects on tissues; but his theory found no echo in the medical world of his time.

Even after physicians had accepted the germ theory, they had difficulty in apprehending how microscopic creatures could ever cause all the destructive lesions or dramatic physiological disturbances observed in diseases of animals or man. Until one had become used to the idea, there was indeed something incongruous in the thought that a minute bacillus could kill an ox or a horse. Pasteur, thanks to his great chemical and physiological experience and imagination, was not disturbed by this apparent discrepancy. He postulated that much of the pathology of anthrax might be due to the fact that *Bacillus anthracis* competed successfully with tissue cells for oxygen, and perhaps also for other metabolites. Then, three years later, he made the funda-

mental discovery that the toxic manifestations of fowl cholera could be produced by injecting into normal animals a bacteria-free filtrate of culture of the causative agent. Perceiving that this discovery brought him close to the biochemical basis of disease, he formulated the project to isolate the toxin and study it chemically, but circumstances prevented him from carrying out this program (reviewed by Dubos, 1950).

It is only during very recent years that some progress has been made toward the understanding of the toxemia and pathology of infection, that we have come to learn the nature of the "disagreeable Parts" of microorganisms which Benjamin Marten postulated to be "inimicable to our Nature." It seems certain that their "peculiar Shape" is of no relevance to the production of disease, for the harmful effects of microorganisms very seldom, if ever, have a purely mechanical cause. It is true that in animals suffering from anthrax death appears to be related to blockage of the capillaries by emboli caused by the bacillary bodies, but it is probable that these emboli are secondary to other toxic effects on blood cells or on the vascular endothelium.

The constituents and products of microorganisms responsible for the pathological effects of infection include substances of many various chemical types — proteins, lipids, polysaccharides, as well as a multiplicity of smaller molecules. They differ profoundly in the mechanism of their action and affect virtually every function and structure of the body. Some of them exhibit a clearly defined primary toxicity; others merely set in motion tissue processes which eventually result in secondary pathological manifestations. Some bring about metabolic disturbances resulting in systemic effects; others, reactions which are primarily destructive of tissues. In fact, so varied, and so often undefined, are microbial toxins in composition and in the mechanism of their effects that it will probably never be possible to classify them according to an orderly sytem. Their chemical and biological characteristics have been described recently in a critical and comprehensive review (van Heyningen, 1950). Table 36, taken from

TABLE 36

Characteristics of a few toxic biological substances

Substance	Number of MLD (kg animal) per		Animal species
	μg toxin	μM toxin	
Aconitin	0.077	60	Rabbit
Ricin	.013	1,120	Mouse
Cobra neurotoxin	.9	90,000	Mouse
Urease	.013	1,300	Rabbit
Welch alpha toxin	.4	40,000?	Mouse
Typhoid toxin	.0002	?	Mouse
Diphtheria toxin	3.5	245,000	Guinea pig
Shiga neurotoxin	?	100,000,000	Mouse
Tetanus toxin	1200	120,000,000?	Guinea pig
Botulinus A toxin	1200	1,200,000,000	Guinea pig
Botulinus B toxin	1200	72,000,000	Guinea pig

Modified from van Heyningen, 1950.

this review, presents the approximate molecular weight and biological activity of a few bacterial toxins in comparison with the most toxic organic substances of nonmicrobial origin.

Merely for the sake of convenience, and at the risk of much artificiality, we shall attempt to group in the following sections some of the biologically active components and products of microorganisms according to an arbitrary system based primarily on the type of physiological and biochemical disturbance that they produce *in vivo*.

TOXICITY RESULTING FROM METABOLIC COMPETITIONS

While proliferating *in vivo*, microorganisms necessarily release a variety of catabolic products which presumably affect the nutrition of tissue cells. It is also likely that they can remove from the environment certain metabolites important in animal physiology, since the bacterial surface is strongly negatively charged and therefore capable of adsorbing large amounts of inorganic ions as well as of organic complexes. Viruses also have the power

to adsorb, and indeed to bind firmly, a variety of ions and even of active enzymes from the surrounding fluid (Hoagland *et. al.*, 1942; Hoagland, 1943). Finally, the synthesis of microbial protoplasm entails the utilization of many metabolites, some of which may be essential for the maintenance of the normal activities of tissues.

Metabolic interference or competition may express itself merely in reversible inhibitions and need not be of such severity as to result in lethal effects. But even reversible metabolic blocks may be sufficient to alter for a time cellular permeability and functions in such a manner as to cause indirectly a variety of toxic phenomena either general or local in character. Although no experimental evidence is available to evaluate the role in infection of this type of metabolic competition between parasite and host, there are some clinical observations suggestive of its importance.

Most of the manifestations of acute toxicity in pneumococcus lobar pneumonia disappear within a few hours after administration of penicillin. There is no evidence that the drug has any direct antitoxic properties, and moreover the pneumonic lesion retains its pathological characteristics after the discontinuance of therapy and cessation of toxemia. On the other hand, penicillin suddenly interrupts microbial multiplication and it seems not unlikely therefore that some at least of the toxicity of pneumonia is to be traced to the metabolic activities of pneumococci during the spreading phase of the disease. Bacteriostasis caused by an antimicrobial drug, or by phagocytosis mediated through specific antibody (as in serum therapy, or during natural crisis), would then be sufficient to stop the manifestations of acute toxicity. It is likely that part of the rapid symptomatic improvement in tuberculosis which sometimes follows the administration of streptomycin or isoniazid may be credited to a similar mechanism.

There may be cases where the toxic effects of infection are caused by antimetabolites produced by the infectious agent. For example, the etiological agent of wild fire of tomato, *Pseudomonas tabaci*, produces a toxin capable of reproducing in normal tobacco

plants the symptoms and pathology of the disease. Acid hydrolysis of the toxin yields a neutral amino acid (tabtoxinine) which is a derivative of diamino-dipimelic acid. Tabtoxinine can be regarded as a structural analogue of methionine — the methyl SH group being replaced by a 3-carbon chain containing various reactive groups. This similarity in structure has led to the suggestion that tabtoxinine, which accounts for more than half of the amino acid isolated from the toxin, acts as antimetabolite by virtue of its molecular configuration (Woolley, Schaffner, and Braun, 1952). It is worth mentioning in passing that alpha-epsilon diamino pimelic acid, first recognized as a component of tubercle and diphtheria bacilli, is now known to occur in several microbial species (Work and Dewey, 1953). Its possible role as a determinant of toxicity has not yet been investigated.

During recent years, evidence has been accumulating that Rickettsiae and at least some of the viruses can exert a cytotoxic effect independent of their multiplication in host cells. Indeed, injection of large amounts of these agents can cause rapid death of experimental animals and chick embryos. In some cases (for example, that of Newcastle virus in the mouse lung), it seems possible that the toxicity is due to the ability of the virus, or its degradation products, to combine firmly with some cellular constituent, thus removing it from the metabolic pool of the cell and bringing about injury by an indirect interference with metabolism (Davenport, 1952; Ginsberg, 1951, 1954). We have already mentioned that some viruses can firmly bind *in vitro* a number of inorganic and organic substances important in animal metabolism Hoagland *et al.*, 1942; Hoagland, 1943).

It is the study of diphtheria intoxication which has provided the most suggestive evidence that certain toxins can act as metabolic antagonists. The purest preparations of diphtheria toxin available show it to be a protein of molecular weight 72,000 with nothing obvious in its chemical or physical properties to account for its biological activity. However, there are some clues to the possible mechanism of its toxic properties in man and ani-

mals (Pappenheimer, 1947*a*, *b*). As already mentioned, an excess of iron in the culture medium interferes with the production of toxin by *Corynebacterium diphtheriae* (see page 61). When toxin is produced, on the other hand, there is released simultaneously in the culture medium a porphyrin pigment which is either hematoporphyrin (Pappenheimer, 1947*a*, *b*) or coproporphyrin III (Gray and Holt, 1947). The appearance of toxin and porphyrin is inhibited simultaneously by concentrations of iron greater than 100 μg per liter of medium (which do not inhibit the growth of the bacillus). Moreover a remarkable proportionality exists between the concentrations of toxin and of porphyrin pigment released. When iron is added to the medium in excess of the optimal concentration, it is found that every four atoms of the metal added inhibit the production of four molecules of porphyrin and one of toxin (Tables 37–39). As more iron is taken up by the dacilli, these produce instead of toxin greater concentrations of a

TABLE **37**

Effect of iron on growth and metabolic products of *Corynebacterium diphtheriae*

Iron added (gm atoms $\times 10^7$)	Final pH	Total growth * (mg bacterial N)	Precipitable protein from culture supernatant * (mg N)	Bacterial iron content * (mg per gm bacterial N)	Porphyrin † (gm mole $\times 10^7$)	Toxin (gm mole $\times 10^7$)	Toxin nitrogen (percent precipitable protein N)
0	6.65	204	22.7	0.16	32.1 (35.8)	7.6, 7.45	38.2
2.7	6.76	211			30.5	6.95, 7.15	
5.4	6.85	207	24.0		25.1	6.4, 6.4	30.7
8.1	6.68	216	24.8	0.38	22.8	5.5	25.5
10.8	6.94	212	25.2	0.45	19.5	4.95, 4.75	22.0
13.4	6.93	224	26.4		18.1	4.07	17.8
18.8	6.68	226	26.7	0.57	11.7 (15.4)	2.98	12.8
30.4 ‡		241	30.0	0.85	0 (3.7?)	0	0

From Pappenheimer and Hendee, 1947.

* Average of duplicate determinations.

† The values in parentheses were obtained by ether extraction of culture filtrates. The other figures in this column are mean values for change in porphyrin concentration. For simplification we have arbitrarily assumed a blank of 3.7×10^{-7} moles of porphyrin when 30.4×10^{-7} gm atoms of iron have been added.

‡ In this line the values are calculated for complete toxin inhibition.

TABLE **38**

Effect of iron on succinate oxidation by *Corynebacterium diphtheriae*

Iron added (μg/300 ml of medium)	Park-Williams No. 8 (Toronto) strain, *intermedius*		Halifax strain, *gravis*
	Toxin (Lf per ml)	Q_{O_2} (mm^3 O_2 per mg N per hr)	Q_{O_2} (mm^3 O_2 per mg N per hr)
0	70	20–30	81
20	62	35–50	
50	48	43–66	82
70	40	72	
100	25	110	129
200	0	106–120	137

From Pappeinheimer and Hendee, 1947.

TABLE **39**

Effect of iron on catalase production by *Corynebacterium diphtheriae*

Iron added (μg/300 ml of medium)	P–W No. 8 (Toronto) Strain	
	Growth (mg N)	Catalase (Kat. f)
0	162	185
50	215	400
100	228	610
200	240	840

From Pappenheimer and Hendee, 1947.

respiratory pigment which appears to be cytochrome *b* or a closely related conjugated protein. Since cytochrome *b* contains four heme radicals per molecule, it follows that diphtheria toxin might be the protein moiety of diphtherial cytochrome *b*. According to this view, the bacilli grown in media deficient in iron would still synthesize the protein moiety and the porphyrin, but fail to combine them together into cytochrome *b*.

This hypothesis could be proven directly if it were possible

to prepare the toxin from diphtheria cytochrome *b*, or if cytochrome *b* could be prepared from the toxin by addition of porphyrin and iron. But neither of these critical operations has yet been achieved. In the meantime, additional indirect evidence in favor of the hypothesis has been derived from metabolic studies on the *Cypropia* silkworm (Pappenheimer and Williams, 1952). Advantage was taken of the fact that the amount and distribution of cytochrome in this insect and the importance of the cytochrome system for its metabolism differ widely according to its developmental stage. This was demonstrated by measurement of cytochrome *c*, succinic dehydrogenase, and cytochrome oxidase in the prepupal, pupal, and adult phases of the insect.

The cytochrome system is extremely deficient during the dormant (pupal) stage of *Cypropia* but increases progressively during development. Interestingly enough, the susceptibility of the insect to the injection of diphtheria toxin follows a parallel course; it is extremely low during the dormant stage but increases progressively as development proceeds. Moreover, injection of 1 μg of toxin at any given time results in immediate cessation of development and of synthesis of cytochrome *b*. It is of particular significance, furthermore, that the different anatomical parts of the pupa are not equally deficient in cytochrome *b* nor equally resistant to the toxin. When large amounts of diphtheria toxin are injected into the insect during the dormant stage, the heart muscle, which contains almost no cytochrome, continues to beat normally for long periods of time, whereas, in contrast, the intersegmental muscles, which alone of the pupal tissues are rich in cytochrome, soon stop functioning and undergo necrosis.

All these findings have led to the hypothesis that diphtheria toxin blocks the synthesis of cytochrome *b* or of a closely related enzyme in susceptible animals. One need only assume that the toxin resembles the protein moiety of the animal's cytochrome *b* closely enough to compete with it in the cytochrome-synthesizing system, but not sufficiently to be incorporated into an active cytochrome suitable to the animal economy. This hypothesis

would be compatible with the fact that diphtheria intoxication develops slowly even in the most susceptible animals. Whatever the amount of toxin injected, it would be necessary that the store of cytochrome *b* in the animal be depleted before interference with its synthesis manifested itself by metabolic difficulties.

Although tetanus toxin, the several botulinus toxins, and Shiga neurotoxin differ greatly from diphtheria toxin in their pharmacological properties, they have some features in common with it. As we shall see (pp. 80–81) they too are proteins capable of causing injury to tissues in doses containing a relatively small number of molecules, and they exhibit a prolonged latent period during which no lesions or symptoms can be demonstrated. Their latent period and extraordinary potency suggest that they do not act as enzymes themselves nor by direct inhibition of existing enzymes, but rather by interfering with the synthesis of some essential cellular constituent. These characteristics are quite in contrast with those of the enzyme-toxins which we shall now consider.

ENZYME TOXINS

The lecithinase of *Cl. welchii*, first recognized under the name of alpha toxin, is now regarded as the chief cause of toxemia in gas gangrene. In the presence of calcium, it is capable of hydrolyzing lecithin rapidly (into an ether-soluble stearyloleylglyceride and acid-soluble phosphocholine), and sphyngomyelin slowly; it does not attack kephalin, glycero phosphocholine, lysolecithin, or cerebrosides (Macfarlane and Knight, 1941; Macfarlane, 1942, 1948).

Alpha toxin is lethal for mice and other laboratory animals. Its inhibitory effect on cytochrome oxidase appears sufficient to account for the signs of toxemia in clinical gas gangrene (Macfarlane, 1950*a*). It can also cause lysis of erythrocytes of certain animal species and necrosis of other types of cells. Its action is extremely rapid, there being no appreciable latent period be-

tween injection and onset of symptoms — a characteristic which differentiates it from the classical exotoxins.

Studies with horse and sheep erythrocytes have revealed that their hemolysis by alpha toxin is always preceded by decomposition of some of the phospholipids of the cell, indicating that hemolysis is primarily dependent upon the lecithinase activity of the toxin preparation. The *Cl. welchii* lecithinase is inhibited by antitoxic sera prepared against *Cl. welchii* alpha toxin. Titration of a number of antisera using as criteria their ability to neutralize the lethal effect of the toxin for mice, its hemolytic power, and lecithinase activity, yield very good agreement between the values obtained in the different tests (Macfarlane and Knight, 1941; Roth and Pillemer, 1953). All evidence points to the fact, therefore, that lecithinase and alpha toxin are one and the same substance. Needless to say, *Cl. welchii* produces in addition to the lecithinase other biologically active substances, collagenase and hyaluronidase among many, which also contribute to the toxemia of gas gangrene. However, the fact that sera directed against the lecithinase exert a protective effect against experimental infection and have therapeutic value in the treatment of gas gangrene, whereas anti-collagenase and anti-hyaluronidase sera are ineffective in these respects, gives weight to the conclusion that alpha toxin is of unique importance in the pathology and symptomatology of *Cl. welchii* infections.

Lecithinases are produced by many groups of microorganisms other than clostridia, and also by tissue cells of various origin. But it must be emphasized that not all lecithinases possess identical toxic activity. In fact, marked differences can be recognized even between the various clostridial lecithinases. Thus, whereas the alpha lecithinase of *Cl. welchii* will hemolyse sheep erythrocytes and not horse erythrocytes, the lecithinase of *Cl. oedematiens* will hemolyse horse but not sheep erythrocytes (Table 40). Yet lecithin is present in sheep and horse cells, both types of enzymes hydrolyse the same chemical bond, and both need calcium to exert their enzymatic action. The hemolytic

TABLE **40**

Lecithinase activity on sheep and horse erythrocytes of two clostridial toxins

Time (min)	Decrease in lipid P, erythrocytes incubated with:			
	Cl. welchii toxin		*Cl. oedematiens* toxin	
	Sheep (μg)	Horse (μg)	Sheep (μg)	Horse (μg)
30	10	4	0	22
45	18	11	0	25
60	24	21	0	26

From Macfarlane, 1950*b*.

selectivity of the toxins must therefore reside in some fact other than enzyme activity, perhaps the accessibility of the substrates to enzymatic action (Oakley, Warrack, and Clarke, 1947). This is apparent from another study which revealed that the rates of hydrolysis of the phospholipids in intact erythrocytes of horse and sheep by three immunologically distinct lecithinases of *Cl. welchii* and *Cl. oedematiens* are strikingly different. These rate differences appeared sufficient to account for the comparative behavior of horse and sheep erythrocytes toward the toxins (Macfarlane, 1950*b*). Similarly, equipotent amounts of lecithinase (as tested *in vitro* under standard conditions) obtained from three different strains of *Cl. bifermentans* proved, respectively, 9, 60, and 75 times less toxic than the corresponding amount of *Cl. welchii* lecithinase (Table 41; Miles and Miles, 1950). While it is probable that this selective action of lecithinases on the erythrocytes of different species is related in some way to the problems of virulence and biological specificity, nothing is known of the factors peculiar to each strain which determine this selectivity.

In addition to lecithinase, there are many other microbial enzymes which are released in an active form during infection. We can mention only a few of those which may play some part in the pathogenesis of disease.

Hyaluronidase is produced by many bacterial species. It was

TABLE 41

Comparison of five crude clostridial lecithinases

Strains	Lecithinase	Hemolysis (mouse erythroc.)	LD_{50} (mouse)
Cl. bifermentans No. 17	100	173	ca. 4300
Cl. bifermentans No. 18	100	120	ca. 5400
Cl. bifermentans No. 20	100	28	670
Cl. welchii No. 290	100	315	59
Cl. welchii Pool	100	218	85

From Miles and Miles, 1950.

first described under the name of spreading factor because of the fact that it facilitates the spread of infective inocula through the area of the skin in which it is injected (Duran-Reynals, 1942). This property is an expression of its ability to hydrolyse hyaluronic acid, a highly viscous amino-polysaccharide acid which is a constituent of the intracellular matrix or ground substance (reviewed in Duran-Reynals, 1950).

Collagenase is known to be formed only by some strains of clostridia; it can bring about disintegration of collagen fibres.

The cathepsin of group A streptococci, as well as other proteolytic enzymes, has recently been shown to produce characteristic myocardial necrosis when injected intravenously into rabbits (Kellner and Robertson, 1954).

Desoxyribonuclease (dornase), produced by hemolytic streptococci and to a lesser extent by bacteria of other species, depolymerizes desoxyribonucleic acid. When released from tissue cells and especially from leucocytes as a result of their necrosis, this acid imparts to cellular exudates a viscous, tenacious character which is readily broken down by dornase (Sherry and Goeller, 1950; McCarthy, 1948).

Streptokinase is a product of group A streptococci which converts the protease precursor in plasma (plasminogen) into its active proteolytic form plasmin, and thus renders it capable of causing the dissolution of fibrin clots (Christensen, 1954). In-

jection of streptokinase into rabbits can bring about myocardial lesions similar to those produced by various proteolytic enzymes (Kellner and Robertson, 1954). The failure of streptokinase to cause anaphylactoid reactions is discussed on page 91.

Staphylocoagulase is produced by virulent staphylococci. In contrast to streptokinase, it intervenes in the blood clotting mechanism in such a manner as to produce a thrombinlike substance which causes rapid coagulation of fibrinogen even in citrated plasma (Duthie and Lorenz, 1952).

Many purified virus preparations possess various types of enzymatic activity. But it is not yet known whether the enzymes concerned are an intrinsic part of the virus particles themselves or only adsorbed on them from the cellular environment in which they grow (Hoagland *et al.*, 1942, 1943; Pirie, 1947, 1953). On the basis of present evidence, it appears, however, that at least one of these enzymes is really part of the structure of certain viruses, of influenza virus, for example. This enzyme acts on the virus receptors of erythrocytes and is thought to lead to the elution of virus particles from the component (receptor) of erythrocytes to which they appear to be bound. Not only does this enzyme act on erythrocytes, but also it acts on mucoproteins which react with the virus. Such mucoproteins contain the newly discovered amino acid, 2-carboxypyrrole, which may be the key point of virus attachment. The mucoprotein appears to be present on the surface of the respiratory epithelium and other types of susceptible cells, and exists in a free form in certain body fluids. It is not yet clear what bearing the enzymatic destruction of the receptors of susceptible cells by the virus particle has on the course of infection (Hirst, 1943; Gottschalk, 1954; Tamm and Horsfall, 1952; McCrea, 1953).

All the microbial enzymes that we have just briefly considered take a direct or indirect part in pathological processes, but — except for the lecithinases of clostridia — their effects are difficult to evaluate because they do not cause obvious toxic reactions. It is important to notice that they do not kill the tissue cells

which they attack and that some of them (for example, hyaluronidase, collagenase, dornase, fibrinolysin) appear to hydrolyse only substrates present in the extracellular environment. As a consequence, their toxic effects are only those of the split products of their specific substrates and are not apparent unless large amounts of enzymes are involved. It is probable that they affect the course of infection only indirectly by changing the physical and chemical characteristics of the environment surrounding the parasite.

GENERAL HISTOTOXICITY *VERSUS* SELECTIVE TOXICITY

Among microbial products, there are some which affect a great variety of tissue cells, and others which are extremely selective in their action (Table 42). To the first group belong diph-

TABLE **42**

Toxicity of highly purified bacterial toxins.

Toxin	General Histotoxicity	LD_{50} (mg/kg animal)	Animal
Botulinus Type A	No	620,000	Mouse
		1,200,000	Guinea pig
Tetanus	No	200,000	Mouse
		1,200,000	Guinea pig
Shiga neurotoxin	No	750	Mouse
		1,150,000	Rabbit
Diphtheria	Yes	3	Mouse
		3,500	Guinea pig
Welch alpha toxin	Yes	400	Mouse

therial toxin and the clostridial lecithinase which have been considered in preceding chapters (pages 69–70, 73–75). As we have seen, they possess certain biochemical characteristics which are clearly responsible for their general histotoxicity — diphtheria toxin interfering probably with the synthesis of cytochrome *b* which is a widespread respiratory pigment, lecithinase destroying phospholipids which are structural components of most cells.

We shall now consider a group of toxins produced by *Cl. welchii* (theta toxin), *Cl. tetani* (tetanolysin), *Clostridium hystolyticum*, *Diplococcus pneumoniae* (pneumolysin), and *Streptococcus pyogenes* (streptolysin O), all of which bring about the rapid lysis of many types of cells, particularly of erythrocytes. All these toxins appear to be proteins active only in the reduced state; they are reversibly inactivated by mild oxidizing agents and are called O hemolysins to denote their oxygen lability; the hemolytic activity of all of them is inhibited by cholesterol and by antibody produced against any one of them.

In addition to its cytolytic activity, streptolysin O (and probably also the other O hemolysins) exerts a marked cardiotoxic action in the intact animal. Peculiar phenomena come to light when this action is studied in the isolated frog's heart. Whereas a single dose of streptolysin O has no visible effect on this preparation, the heart immediately stops in systolic contracture if it is washed after the first dose of toxin and is then subsequently treated with a second dose. This result is not due to the summation of the two amounts of toxin, but to the fact that the first dose brings about the release from heart tissue of a substance which inhibits the cardiotoxic effect. It is the washing away of this inhibitor which renders the preparation susceptible to a second dose of toxin. The inhibitor, which is heat-stable, nondialysable, and chloroform-soluble, resembles cholesterol, but its identity has not been convincingly proven (Bernheimer and Cantoni, 1947).

Mice injected with sublethal doses of streptolysin O develop marked resistance to subsequent injections. This resistance appears within a few hours and lasts for some 30 hours. Of special interest is the fact that it is effective not only against streptolysin O but also against saponin, the cytolytic action of which is also inhibited by cholesterol. There is much reason to believe that the resistance induced by repeated injection of streptolysin O is in some way associated with the release of cholesterol-like substances from the animal's own tissues. Indeed, it is known that

mice can be protected against the lethal effect of streptolysin O by previous injection of cholesterol (Hewitt and Todd, 1939), and a protective effect can be obtained also by using instead of cholesterol the inhibitor released by washing frog's heart after treatment with streptolysin O. Not all sterols, but only those with a certain molecular structure related to that of cholesterol, have the capacity to inhibit streptolysin O. There is good evidence that this inhibition depends upon a binding of the sterol to the lysin and not upon its adsorption on the red cell envelop (Howard, 1953; Howard, Wallace, and Wright, 1953).

Thus, it appears as if a number of microbial products could exert cytotoxic effects through a mode of action which in some obscure manner resembles that of saponin. These O hemolysins stand in sharp contrast to another group of hemolytic bacterial products (the alpha toxin of *Cl. welchii*, the staphylococcus hemolysins, and streptolysin S) which are unaffected by oxygen, and are not inhibited by cholesterol (Bernheimer 1948, 1954).

The various botulinus toxins, tetanus toxin, and Shiga neurotoxin provide good examples of bacterial products exhibiting marked selectivity as to the tissues which they poison. Some of the characteristics of this group will be illustrated by describing experimental results obtained with botulinus A and Shiga toxins.

The type A botulinus toxin has been obtained in a crystalline form as a protein of molecular weight 900,000 and is apparently the most toxic substance known; 1 mg of it contains 1,200,000,000 MLD per kg of guinea pig or almost 10^{17} MLD per mole. It is thought that it acts primarily on the nervous system, the toxic dose having been estimated as 4–8 molecules per nerve cell. The toxin is without action on the adrenergic nerves and is specifically active on the fibers of cholinergic peripheral motor nerves. It seems to act on the motor-nerve endings and to interfere with the release (but not the synthesis) of acetylcholine (Ambache, 1949; Burgen, Dickens, and Zatman, 1949; Stover, Fingerman, and Forester, 1953). Its action is extremely slow as commonly observed in patients (several days incubation period)

and as demonstrated by the use of nerve-diaphragm preparations of rat tissue. In experiments in which such a preparation was treated with 40–200 rat LD_{50}, it took 25–40 minutes before the tension slowly declined, to end in complete paralysis in 70–100 minutes. The latent period was only slightly shorter and the rate of paralysis slightly accelerated with 800–1600 LD_{50}.

The production of Shiga neurotoxin, like that of diphtheria toxin, is in some manner inhibited by the presence of excess of iron in the medium, even though this metal is essential for the growth of *Shigella shigae*. When produced and purified under the proper conditions, Shiga neurotoxin can be obtained as an electrophoretically homogenous protein possessing a toxicity for the rabbit of the same order as the toxicity of tetanus and botulinus toxins for the guinea pig (Van Heyningen and Gladstone, 1953).

Despite the fact that the lesions most commonly observed following administration of Shiga neurotoxin are in the intestinal mucosa, there is good evidence that the toxin acts via a mechanism located in the central nervous system. Direct contact between the toxin and intestinal mucosa of dogs produces no lesion in the exposed loop, whereas injection of the toxin by the intravenous route causes the appearance of a number of focal lesions not associated at first with any visible alterations in the structure of the vascular walls. The tissue edema and extravasation of the blood cells around the lesions reveal changes in permeability of the vascular bed, probably due to the occurrence of vasospasm in the involved areas. These phenomena can be inhibited by injection of tetraethyl ammonium chloride. Since this drug interferes with the passage of autonomic impulses through sympathetic ganglia, it appears that Shiga neurotoxin has its primary action in the central nervous system itself. It is of interest that paralysis of the sympathetic nervous system at the myoneural junction or in the ganglia by means of drugs prevents the tissue changes caused by the toxin, as well as the hyperglycemia and hemoconcentration characteristic of the sympatho-mimetic response (Penner and Klein, 1952).

THE PARTICIPATION OF TISSUES IN THE TOXICITY OF GRAM-NEGATIVE BACILLI

From the cells of most, if not all, Gram-negative bacilli in the "S" phase, one can separate toxic fractions (endotoxins) usually associated with lipid-polysaccharide-peptide complexes. Although endotoxins (or at least the complexes with which they are associated) exhibit sharp specificities in serological tests, they all bring about the same pathological effects. The most characteristic signs in mice are diarrhea and intestinal congestion, followed in a few hours by death if a large enough dose of toxin is injected. Other signs include hypo- and hyperglycemia, changes in blood inorganic phosphate levels, hyper- and hypothermia, leucopenia, and endothelial damage resulting in hemorrhages. Since most toxicity tests have been carried out with crude fractions, it is impossible to say whether these symptoms are produced by one or several different components of the Gram-negative bacterial cells.

The fundamental similarity in the toxic action of endotoxins also comes to light when attempts are made to vaccinate against them. Whatever the technique of vaccination used, the resistance elicited is never of a high order, nor does it last long, even though it is easy to obtain sera with high titers of precipitating antibodies. Moreover, the serum of the resistant animals has little if any ability to confer passive antitoxic protection. On the other hand, the increased resistance achieved by vaccination is effective not only against the toxin of the bacterial species used as antigen, but also against extracts of other Gram-negative bacilli. Indeed, there is evidence that white rats can be protected against the toxic effects of heat-killed suspensions of *Rickettsia prowazeki* by repeated injections not only of these organisms, but also of Gram-negative bacilli. This finding acquires significance in view of the fact that the toxicity of Rickettsia manifests itself by hypothermia, lowering of glycogen level, and other reactions similar to those elicited by Gram-negative toxins (Olitzki, Czaczkes, and Kuzenok, 1946). Because of these immunological peculiarities,

the resistance elicited by injection of Gram-negative bacilli or their endotoxins is often referred to as "tolerance" rather than as immunity.

We cannot review here the immense body of detailed observations — as yet not integrated — which have been made concerning the mode of action of these endotoxins. Instead, we shall discuss only some aspects of the problem which illustrate the participation of tissue factors in the manifestation of their toxicity.

Pyrogenic activity. The pyrogenic activity of Gram-negative toxins has been the subject of particularly intensive study. In man, the injection of amounts as small as 0.1 μg of purified typhoid toxin is capable of producing a marked febrile response (Favorite and Morgan, 1942). But patients receiving multiple intravenous injections of the toxin, or of crude vaccine, develop remarkable tolerance to this pyrogenic effect, becoming capable of withstanding without appreciable reaction several hundredfold the dose toxic to a normal individual (reviewed by Bennett and Beeson, 1950). Like man, normal rabbits and dogs respond with fever to the injection of small amounts of Gram-negative toxins. They too can acquire rapidly a high degree of nonspecific tolerance to the pyrogen, and this tolerance appears to involve a change in the functional activity of the reticulo-endothelial system providing for a more rapid disposal of foreign material (Beeson, 1947).

When additional amounts of pyrogen are injected into animals rendered tolerant by repeated injections, the material disappears from their blood more rapidly than from the blood of normal animals. This enhancement of the clearing mechanism is not specific, since rabbits previously injected with typhoid vaccine, for example, have an enhanced ability to remove the pyrogens of *Serratia marcescens* and of *Pseudomonas aeruginosa* from their blood.

Whatever the dose of toxin injected into rabbits, it takes some 30–40 minutes before fever sets in. Yet in rabbits fitted with an

ear chamber permitting direct visualization of the vascular bed, a sticking of white cells can be observed immediately following the injection of toxin (W. B. Wood, personal communication). Another immediate effect of the intravenous injection of bacterial endotoxins into rabbits is a profound leucopenia, primarily a granulocytopenia, which reaches its maximum after 1–2 hours, and is followed a few hours later by leucocytosis (Bennett and Beeson, 1950). At the time of the leucopenia, the pulmonary alveolar capillaries are enlarged and loaded with large numbers of leucocytes, suggesting some alteration of the leucocytes or of the vascular endothelium.

All facts taken together suggest that the febrile response is not due to a direct effect of the toxin on the hypothalamus, but rather to the release of pyrogenic material from the tissues. It is commonly assumed that the active principle is a product of cellular breakdown, probably of leucocytes. During recent years, however, some evidence has been gained that the pyrogenic effect involves a prior reaction, not necessarily with cells, but with a plasma constituent, as shown by the following facts.

When heat-killed typhoid bacilli are incubated with citrated plasma before injection, the febrile response of the injected animal occurs much sooner and is more intense than in animals receiving the vaccine incubated in saline instead of plasma (Le Quire, 1951). In experiments with *Proteus vulgaris*, the blood of normal rabbits having received this material intravenously was found to remain capable of producing a febrile reaction in normal recipient rabbits for several hours thereafter. The fever appeared sooner following injection of febrile blood withdrawn 40–180 minutes after administration of the toxin to the donor than following injection of blood withdrawn earlier. Moreover, the latent period was shorter than that following direct injection of even larger amounts of pyrogen into a normal animal. These facts are compatible with the view that interaction between pyrogen and plasma results in the formation of an "endogenous pyrogen" (Grant and Whalen, 1953). Preliminary information recently

published indicates that the development of tolerance to bacterial pyrogen might be due to some change in the blood preventing or retarding the reaction between pyrogen and plasma assumed to be responsible for production of fever (Grant, 1953). Available data do not permit relating this hypothesis to the detoxifying role of reticulo-endothelial cells (page 83) (Beeson, 1947).

It is known that pyrogenic materials are often released at the site of inflammatory reactions, and the name "pyrexin" has been given to a fever-inducing fraction separated from sterile exudates (Menkin, 1940). It is possible that the latent period, before the pyrogenic effect of bacterial endotoxins becomes manifest represents the time required for the liberation of a pyrexin-like substance from tissues — either from injured leucocytes or from the plasma (for a contrary view see Bennett and Beeson, 1953, and Harris, 1953).

Shwartzman phenomena. The most extensively studied of the toxic manifestations of Gram-negative toxins are those occurring in rabbits treated to elicit the Shwartzman phenomena, either local or general (Shwartzman, 1937). As is well known, the local Shwartzman reaction is induced by injecting a first "preparatory" dose of toxin intradermally into rabbits and a second "provocative" dose (not necessarily derived from the same bacterial species) intravenously a few hours later (8–30 hours preferably). Within a short time after the provocative injection, gross hemorrhage and then necrosis develop at the preparatory site. The generalized Shwartzman reaction is elicited by two intravenous injections of toxic material 24 hours apart. Its most characteristic result is bilateral cortical necrosis of the kidneys (reviewed in Thomas and Good, 1952).

It is obvious that the Shwartzman reaction, either local or general, is the expression of intense vascular damage, but the following facts suggest that this damage is an indirect rather than a direct effect of the toxin.

The Shwartzman reaction cannot be induced in rabbits which have been treated with nitrogen mustard, or benzene, or x-radia-

tion, shortly before the preparatory dose. These treatments leave unaffected other effects of the toxin, its pyrogenicity and lethal power, for example, and there is reason to believe that they inhibit the reaction through their leucopenic effect (Bennett, 1952*a*, *b*; Schlang, 1952; Bennett and Cluff, 1952; Stetson and Good, 1951).

Several hypotheses have been formulated to explain the pathogenesis of the necrotic reaction. It seems certain that hemorrhagic infarction is one of its fundamental causes, since repeated injections of large doses of heparin prevent not only focal dermal necrosis, but also the bilateral renal necrosis of the systemic Shwartzman reaction. In contrast, heparin fails to inhibit the leucopenic, pyrogenic and lethal effects of the toxin (Good and Thomas, 1953; Cluff and Berthrong, 1953*a*, *b*). Although the role of leucocytes in the causation of thrombosis is suggested by failure of thrombi to form when the animal is rendered leucopenic, it must be kept in mind that the methods used to produce leucopenia have also ill-defined but important effects on blood platelets and other factors involved in blood coagulation (Stetson and Good, 1951).

There is one further aspect of the activity of leucocytes which may play an indirect part in the genesis of the necrotic hemorrhagic lesion. The areas of rabbit skin which receive the preparatory dose of endotoxin consistently exhibit a striking metabolic abnormality, resulting in the local accumulation of large amounts of lactic acid (Thomas and Stetson, 1949). The glycolysis in the prepared skin sites is due to the influx of exudate polymorphonuclear leucocytes, rather than to a direct alteration of the metabolism of intrinsic skin elements by the intradermally injected toxin (Tables 43 and 44). Treatment with nitrogen mustard inhibits accumulation of lactic acid in the prepared skin site, very likely because it causes a leucopenia affecting primarily the polymorphonuclear cells (Table 45). On the other hand, rabbits receiving an intravenous injection of toxin become highly susceptible to the local necrotizing effect of certain proteolytic enzymes

injected into the skin, a finding which has led to the view that one phase of the local Shwartzman reaction might depend upon activation of proteases (cathepsins) in the skin under the local

TABLE 43

Effect of meningococcal toxin on production of lactic acid in skin

Rabbit No.	Lactic acid present * at time of removal of:		Lactic acid formed *in vitro* * in 2 hr at 37° C by:	
	Normal skin	Prepared skin	Normal skin	Prepared skin
1	1.7	3.56	0.02	9.74
2	0.85	4.0	3.7	23.7
3	2.0	2.75	2.8	10.75
4	1.6	8.8	4.2	14.8
5	1.4	4.5	3.1	11.3

After Thomas and Stetson, 1949.

* Lactic acid content of normal rabbit skin, and of prepared rabbit skin (injected intradermally with meningococcal toxin 18 hr previously), and the amount of lactic acid formed by normal and prepared skin during 2 hr at 37° C in an atmosphere of 95 percent O_2 and 5 percent CO_2. Results expressed as milligrams of lactic acid per gram dry weight of tissues.

TABLE 44

Effect of meningococcal toxin on oxygen uptake and carbon dioxide production by rabbit skin (microliters of gas per gram wet weight per hour)

Skin sample	Rabbit No.	Without glucose			With glucose		
		O_2	CO_2	R.Q.	O_2	CO_2	R.Q.
Prepared	1	288	204	0.71	270	248	0.90
	2	364	222	.61	325	243	.755
	3	402	294	.73	345	301	.87
	4	408	294	.72	374	310	.83
Normal	5	476	260	0.545	475	328	0.69
	6	286	154	.54	295	197	.67
	7	423	297	.70	377	294	.78
	8	384	219	.57	362	254	.71

After Thomas and Stetson, 1949.

TABLE **45**

Effect of treatment with nitrogen mustard on Shwartzman reaction

Treatment of rabbits	Examination	Before skin preparation	After skin preparation
Normal	Leukocytes in blood	103–112 × 10^2	107–125 × 10^2
	Polys. in blood	43–82 × 10^2	32–57 × 10^2
	Local skin site: Polys.		Heavy infiltration
	Local skin site: Lactic acid	1–2 mg/gm	5.5–6.5 mg/gm
	Shwartzman reactivity		Yes
HN_2 treated	Leukocytes in blood	25–28 × 10^2	15–24 × 10^2
	Polys. in blood	0–27	0–19
	Local skin site: Polys.		None or occasional
	Local skin site: Lactic acid	1–2 mg/gm	2–3.5 mg/gm
	Shwartzman reactivity		No

Compiled from data in Stetson and Good, 1951.

conditions associated with glycolysis (Thomas and Stetson, 1949).

The increased susceptibility of the blood vessels in prepared skin sites may be due in part to the large amount of lactic acid accumulating in these areas, this metabolite being known to have a pronounced effect on vascular tonus (Hemingway and McDowall, 1926) and to exert damaging effects on other tissue elements (Reznikoff and Chambers, 1927; Carey and Massopust, 1944). It appears possible that lactic acid inflicts upon the endothelium of the capillaries and veins a type of damage which remains inapparent until the intravenous challenging injection has been given, whereupon it becomes manifest through the occlusion of small vessels caused by the adherence of leucocyte-platelet thrombi to the vascular walls and perhaps through the participation of proteolytic enzymes (Stetson, 1951*a*, *b*).

Inhibition of leucocytic migration. Another type of evidence for the participation of a tissue factor in the genesis of the toxic reactions caused by endotoxins has recently come to light. When leucocytes taken from the buffy coat of the blood of rabbits hav-

ing received an intravenous injection of endotoxin are observed *in vitro* (in tissue cultures or slide-cell preparations), they are found to have lost partially or totally the ability to migrate on plasma clots (Cluff and Berthrong, 1953*a*; Cluff, 1953). Inhibition of migration appears to be the outcome of some change which gives a peculiar stickiness to the cellular surface. As normal migratory activity is restored by centrifuging the cells and resuspending them in a new fluid, one may wonder whether this stickiness is not caused by the adsorption of an abnormal plasma constituent on the surface of the leucocytes (Cluff, 1954, personal communication).

Inhibition of migration following administration of endotoxin presents several of the characteristics already mentioned for the pyrogenic effect and for the Shwartzman reaction. It does not occur when the cells are taken from rabbits which have been rendered tolerant to the toxin by daily injections of it. This tolerance cannot be passively transferred with serum from resistant animals, and is independent of the serological specificity of the bacterial species from which the toxin had been obtained. Although tolerance is demonstrable as rapidly as within 5 minutes after injection of the toxin, leucocytes of normal, uninjected rabbits migrate at a normal rate from the buffy coat into plasma to which toxin has been added *in vitro* — even in amounts much larger than those sufficient to inhibit migration when added *in vivo*. This failure of the toxin to act *in vitro* suggests that its inhibitory effect on migration is dependent in some manner upon a reaction which occurs *in vivo*.

Thus all the toxic manifestations of Gram-negative endotoxins so far recognized appear to be merely secondary effects, but unfortunately nothing positive is known of the nature and site of the primary reaction. Suffice it to mention here in passing that typhoid toxin (as well as others) appears to possess marked affinity for the sympathetic system and that its effect on the vascular bed is mediated through this system (reviewed in Reilly, 1942).

TOXIC MANIFESTATIONS OF ANTIGEN–ANTIBODY SYSTEMS

The toxic effects of antigen-antibody reactions *in vivo* will be illustrated in the following pages by two examples, namely, the anaphylactic shock and the local hemorrhagic necrosis known under the name of Arthus reaction. We shall not describe the immunological or histological aspects of these manifestations of allergy but discuss instead some of their biochemical determinants.

Anaphylatoxins and tissue proteases. It has long been suspected that a variety of substances collectively referred to under the name "anaphylatoxins" are released during anaphylactic shock and play an important part in the production of the anaphylactic syndrome (reviewed by Chase, 1952). There is also evidence of an indirect nature suggesting that anaphylatoxins can be liberated by immune reactions under certain circumstances. A long-held view is that antigen-antibody complexes adsorb some protease inhibitors from serum and thus activate its potential proteolytic system; proteolysis would then result in the liberation of biologically active split products (Bronfenbrenner, 1915). There is in fact no doubt that (*a*) serum contains several types of proteases and protease inhibitors (some of which have been separated in the pure state) (Grob, 1942); (*b*) these inhibitors can be removed or inactivated by several techniques which cause the serum to become proteolytic; (*c*) some of the products of autoproteolysis of plasma and serum are toxic and in particular may give rise to anaphylactoid reactions.

It is not easy to establish that the serum proteolytic system can really be activated by antigen-antibody reactions. Indeed, several groups of investigators have come to the conclusion that it cannot (Jemski, Flinck, and Stinebring, 1953). Recent experiments seem to establish, however, that activation of the profibrinolysin (plasminogen) of guinea-pig serum can be brought about by an antigen-antibody system if the reaction takes place in the presence of another factor which is similar to, or identical

with, serum complement (Geiger, 1952; Ungar, Damgaard, and Hummel, 1953; Table 46). It is worth mentioning in this respect

TABLE 46

Activation of guinea-pig profibrinolysin by Ea–anti-Ea (G.P.) in the presence of complement

Egg albumin immune serum *		Normal serum, fresh (ml)	Fibrinolytic activity found (10^{-3} U/ml serum)
Fresh (ml)	Heated (ml)		
—	—	0.2	12.5
0.1	—	—	102.5
—	0.1	—	20.0
—	.1	.01	62.5
—	.1	.1	92.5
—	.1	.2	130.0

After Ungar, Damgaard, and Hummel, 1953.
* 2 mg of egg albumin added per milliliter of serum.

that active fibrinolysin (plasmin) can inactivate complement (Pillemer *et al.*, 1953), a fact which may account for some of the discrepancies in the results obtained by different investigators.

Thus, it appears likely that under the proper conditions *in vivo* some local activation of serum protease can result from antigen-antibody reaction. It has proved difficult to develop analytical techniques sensitive enough to detect the occurrence of serum proteolysis during anaphylactic shock and to prove its relation to the symptoms. The many complex aspects of this problem have been exhaustively analyzed in a recent review (Burdon, 1953) and cannot be discussed here. Suffice it to state that there is solid ground for the view that certain protein split products, released as a result of activation of serum protease, play an important part in the manifestations of anaphylaxis.

The fact that streptokinase, which is the most potent activator of serum protease known, does not lead to the production of substances capable of eliciting anaphylactic-like reactions either *in vitro* or *in vivo* (McIntire, Roth, and Sproull, 1952), seems to militate against this theory. It must be kept in mind, however,

that several types of tissue proteases can be activated through the removal of inhibitors by immunological phenomena, and that the various products of proteolysis by different enzymes may not all have the same biological activity. It has been recently claimed, for example, that plasmin (resulting from the action of streptokinase on plasminogen) has a somewhat limited enzymatic activity. The products of its attack on fibrinogen and fibrin are of high molecular weight and still precipitable by trichloracetic acid (Kaplan, 1954), and it is possible that they possess little anaphylactoid activity. There is evidence, on the other hand, that protein split products lose their toxicity as proteolysis progresses *in vitro* (Bronfenbrenner, 1915*a*, *b*, *c*), and consequently that anaphylatoxins may also be inactivated *in vivo* under certain conditions.

Activation of proteolysis may have many effects other than anaphylactic manifestations on the evolution of disease. Destruction of fibrin (either intravascular or in extracellular exudates) or of other protein constituents (perhaps even of collagen) may in certain cases take place and thus have far-reaching influence on the structure and evolution of various types of lesions (see pages 94 and 119).

The Arthus reaction. As is well known, intradermal injection of the homologous antigen in appropriately sensitized animals results in the production of severe hemorrhagic and necrotic lesions in the areas of skin injected (Arthus reaction). A number of histological characteristics and metabolic phenomena appear to be constantly associated with the Arthus reaction and are probably responsible for some of its manifestations (Stetson, 1951*a*, *b*). There is a profound leucopenia following shortly upon injection of the antigen. Examination of the injected skin area reveals a perivascular accumulation of polymorphonuclear leucocytes: intense aerobic glycolysis with accumulation of lactic acid (Table 47); leucocyte-platelet thrombosis of capillaries and small veins; hemorrhages from the vessels involved in cellular thrombosis. As these reactions are similar to those associated

TABLE **47**

Aerobic glycolysis in rabbit skin during Arthus phenomenon

Rabbit No.	Time after injection of antigen (hr)	Lactic acid produced *	
		Injected skin (mg/hr)	Normal skin (mg/hr)
1	1	5.37	3.38
	2	8.27	3.38
2	1	4.78	3.36
	2	10.34	3.36
3	3	7.12	2.65
4	3	8.80	2.18

After Stetson, 1951*b*.

* The tissue was incubated for 1 hour in the presence of 200 mg percent glucose in Krebs-Ringer solution, with 100 percent O_2 in the gas phase. The results are expressed as milligrams of lactic acid produced per hour per gram dry weight of tissue.

with the Shwartzman phenomenon (see page 85–88), it has been suggested that the hemorrhagic necrosis in both cases is the final outcome of a systemic process involving alterations in leucocytes and platelets, and of the development of local conditions which render the blood vessels in the injected area vulnerable to cellular thrombosis. Direct injury caused by lactic acid, activation of the tissue cathepsins under the reducing conditions prevailing in the inflammatory area, removal of protease inhibitors by the antigen-antibody reaction — all these are factors which might play a role in the genesis of the hemorrhagic lesion (Stetson, 1951*a*, *b*). Needless to say, the Shwartzman and Arthus phenomena are entirely different in the technique of their production; the analogies between them exist only in the development and pathology of the necrotic reactions.

Recent electron-microscope studies have revealed still another manifestation of the Arthus reaction, namely, the occurrence of

profound changes in the local collagen fibers. The lesion does not consist merely in an alteration of the ground substance in and about the fibril bundles, but involves in fact some of the fibrils themselves, which lose their banding and become swollen, irregular, and dense (Rich, Voisin, and Bang, 1953). The precise mechanism of fibril alteration is still obscure. Collagen is known to be resistant to trypsin, chymotrypsin, and papain, but to be readily attacked by a proteolytic enzyme of clostridia and by pepsin (Neuman and Tytell, 1950). Moreover, collagen fibers disintegrate and are "solubilized" in certain acid solutions. It does not seem impossible, therefore, that the alteration of the fibrils observed in the Arthus lesion may be the result of an attack on collagen by certain tissue proteases activated locally, or by the acid reaction originating from glycolysis in the inflamed area.

Thus, it appears that antigen-antibody reactions *in vivo* can have profound effects on the integrity of tissues, a fact which may provide a basis for some of the so-called collagen diseases, and certainly is of importance in accounting for the instability of certain lesions. More generally, the Arthus-like reactions (as well as the Shwartzman phenomena) illustrate the fact that a wide variety of microbial antigens are capable of setting into motion a train of biochemical processes in which the tissues themselves are the active participants, and which result in toxic effects of importance in the symptomatic and pathological manifestations of disease.

CHAPTER 4

Selected topics in tuberculosis

TUBERCULOUS INFECTION AND TUBERCULOUS DISEASE

Surveys throughout the world have revealed that a very large percentage of human adults are tuberculin positive and therefore have been at some time, and probably still are, infected with tubercle bacilli. Furthermore, over 95 percent of those who give no evidence of past infection become tuberculin positive shortly after receiving the BCG vaccine, evidence of the fact that most men are susceptible to even small infective inocula of attenuated bacilli. There is no doubt, therefore, that the great majority of human beings possess little if any resistance to the first phase of tuberculous infection. Yet even the most pessimistic estimates do not place the number of persons suffering from clinical tuberculosis at a level higher than 50,000,000 for the whole world — a fairly small percentage of those infected. In the United States, slightly more than 1,000,000 persons display roentgenological signs of pulmonary lesions, and some 350,000 suffer from active disease — here again a very small percentage of those positive to tuberculin. Thus, tuberculous infection is communicable in the extreme, but it does not necessarily evolve into overt disease.

While the contrast between tuberculous infection and tuberculous disease is so clear, knowledge of the factors which determine the outcome of the host-parasite relationship in tuberculosis is extremely meagre. It may therefore be worth while, even though chastening, to illustrate by a few examples the range of bacteriological problems that remain to be solved before the

pathogenesis of the disease is satisfactorily understood. We shall begin by discussing the pathogenic properties of some of the strains of tubercle bacilli most commonly used in experimental work.

VIRULENCE OF MAMMALIAN TUBERCLE BACILLI

It is a fact of common observation that virulent tubercle bacilli readily give rise *in vitro* to mutant forms that have lost the ability to cause progressive disease in experimental animals. A few of these strains have been extensively studied, *in vitro* and *in vivo* — particularly BCG, derived from a bovine culture, and the cultures H37Ra and R1Rv, both derived from virulent human cultures. The loss of virulence by these strains, first detected through their inability to cause extensive lesions and death in normal animals, can now be studied by other techniques by which they can be differentiated readily one from the other.

As far as can be judged, H37Ra is the only one of these strains that is truly avirulent, being unable to multiply in rabbits, guinea pigs, or mice, or even intracellularly in tissue cultures (Steenken and Gardner, 1946; Pierce, Dubos, and Middlebrook, 1947; Pierce, Dubos, and Schaefer, 1953; Suter and Dubos, 1951; Suter, 1952*b*). This complete loss of virulence is not the result of a rapid bactericidal effect exerted by either body fluids or monocytes on the cells of H37Ra, for these organisms can be recovered in a viable state from tissues for long periods of time after injection into experimental animals. Loss of virulence is apparently due to the fact that the physicochemical environment *in vivo* is not suitable for their multiplication.

In contrast to H37Ra, the cultures R1Rv and BCG can multiply intracellularly both in tissue cultures and *in vivo*, even when very small infective inocula are used (Steenken and Gardner, 1946; Pierce, Dubos, and Schaefer, 1953; Suter, 1952*b*; Mackaness, Smith, and Wells, 1954). It seems therefore more appropriate to refer to them as "attenuated," rather than "avirulent."

Whereas avirulent mutants of mammalian tubercle bacilli (H37Ra-like) are not frequently recovered from cultures, attenuated strains are extremely common. Their comparative behavior *in vivo* can be evaluated quantitatively in terms of the size, severity, and duration of the lesions which they produce in the skin of guinea pigs or rabbits following intradermal injection, or of the rate and extent of their multiplication in the organs of mice (Suter and Dubos, 1951; Pierce, Dubos, and Schaefer, 1953). When compared according to any one of these criteria, they are found to exhibit very marked, and reproducible, quantitative differences, the strain R1Rv being the least attenuated of those tested in our laboratory. As could be expected, BCG has undergone many variations in the course of its long and varied career. When eight substrains of it obtained from eight vaccine-producing laboratories (four from the United States and one each from France, Denmark, Mexico, and Brazil) were studied in comparative tests, each was found to exhibit a characteristic behavior *in vivo* and *in vitro* (Pierce, Dubos, and Schaefer, 1953). Before discussing the factors of the *in vivo* environment which prevent or limit the multiplication of these avirulent and attenuated strains, it may be useful to review briefly some of the attempts made to identify the properties of the bacilli which play a part in virulence.

When strains of mammalian tubercle bacilli of various degrees of virulence are studied *in vitro*, they are found to differ in some of their morphological characteristics. In the proper media, the virulent bacilli tend to adhere one to the other, and to form long strands (at times referred to under the name of "cords"), giving to the culture a characteristic serpentine pattern. In contrast, a few avirulent cultures (H37Ra-like) grow in a helter-skelter disoriented manner. This contrasting pattern of growth is strikingly reflected in colonial morphology (Middlebrook, Dubos, and Pierce, 1947).

Many attenuated cultures consist exclusively, or predominantly, of organisms growing in long strands. Some of them, R1Rv and several of the BCG substrains, for example, cannot be differen-

tiated from virulent organisms by examination of their colonial forms (Pierce and Dubos, unpublished observations). A few others, however, produce colonies reminiscent of the avirulent type. Thus, while the disoriented growth pattern (of H37Ra-like strains) seems to be correlated with inability to multiply *in vivo* or intracellularly in tissue cultures, it is clear that morphological criteria cannot be used as a guide for the analysis of the mechanism of quantitative differences in virulence between attenuated and virulent cultures.

Cytochemically, the cells of the virulent forms are capable of binding neutral red and are stained red by it, even at alkaline reactions, whereas the cells of avirulent H37Ra-like cultures cannot bind the dye under the same conditions (Dubos and Middlebrook, 1948; Dubos and Suter, 1949). Here again, the attenuated cultures behave like the virulent cultures, most of them being strongly neutral-red positive (unpublished observations).

It must be emphasized again that only very few mutant forms of mammalian tubercle bacilli have been found to grow in a disoriented manner, and to be neutral-red negative. If it were proved that all of these mutants are entirely unable to multiply *in vivo*, it could be concluded that the property responsible for the production of serpentine strands, and for the binding of neutral red, is a necessary condition of virulence. Unfortunately, the observations are too few to permit such a generalization. In any case, it is certain that the reason for the differences of behavior *in vivo* between R1Rv or BCG on the one hand, and the virulent cultures on the other, must be sought in characters other than those considered so far. Before proceeding with the analysis of this problem, it is necessary to discuss some long-known facts and recent claims concerning the toxic properties of tubercle bacilli.

TOXINS AND TOXEMIA IN TUBERCULOSIS

It has long been known that the injection of dead tubercle bacilli, or of some of their products, elicits a variety of toxic

effects in experimental animals. The various tuberculolipids in particular call forth intense tissue responses ranging from productive reaction to caseous necrosis (reviewed in Wells and Long, 1932; Sabin, 1941; Sabin *et al.*, 1931; Rich, 1951; Asselineau, 1952). Furthermore, some of the water-soluble or water-dispersible components of culture filtrates can produce a chronic intoxication resulting in cachexia and death (Pinner and Voldrich, 1931). The most thoroughly studied of the properties of tubercle bacilli concerned in toxemia has been their extraordinary power to elicit allergic reactions, of both the immediate (anaphylactic) and delayed (tuberculin-like) type. While these studies cannot be reviewed here, it seems worth recalling that tubercle bacilli, through their wax fraction, possess the unique property of enhancing the antigenicity of tuberculoproteins as well as of other unrelated substances (Freund, 1947; Raffel and Forney, 1948; Raffel, 1950; Forney, 1954). There is little doubt that allergic reactions play the dominant part in the toxic manifestations of tuberculosis.

During the past ten years, there have appeared new claims that there exist in tubercle bacilli powerful toxins bearing a direct relation to virulence. According to one group of reports, extraction of bacillary bodies with mineral oil releases a lipid fraction more abundant in virulent than in avirulent cultures and possessing extraordinary lethal properties for guinea pigs (Choucroun, 1943, 1947). Confirmation of these findings has not been forthcoming.

It has been stated more recently that extraction with petrolic ether releases from certain strains of tubercle bacilli a toxic lipid fraction which has been designated "cord factor" (Bloch, 1950*a*, *b*). Great interest in this fraction has been aroused by the statement that it possesses a unique combination of properties having a direct bearing on virulence. According to the initial reports, the active substance is: present in large amounts only in virulent bacilli; responsible for their ability to form serpentine strands (hence the name cord factor); abundant only in young cultures (thus accounting for their greater virulence); endowed

with great toxicity as manifested by inhibition of migration of leucocytes and lethal effect in certain strains of mice on repeated injections; most active against the strains of mice susceptible to tuberculous infection.

Needless to say, this extraordinary constellation of properties would give to the so-called "cord factor" a unique place among components of virulent tubercle bacilli and indeed would go far toward accounting for their behavior *in vivo*. Unfortunately, several of the statements made concerning this material appear invalid in the light of recent publications as well as of critical scrutiny of the early reports (Philpot and Wells, 1952; Bloch, Sorkin, and Erlenmeyer, 1953; Noll and Bloch, 1953; Asselineau, Bloch, and Lederer, 1953).

The material first described under the name "cord factor" is not a substance but a mixture of the various bacillary constituents soluble in petrolic ether. The toxic component constitutes at most a minute fraction of it (of the order of 1 percent), and there is no indication that it has anything to do with either virulence or serpentine pattern of growth. There is no evidence that the amount of "cord factor" or of toxic material differs from one strain to the other, or is related to their behavior *in vivo*. As to the biological tests used, their significance is at best difficult to interpret. Inhibition of migration of leucocytes, and production of pulmonary lesions in mice, can be brought about by petrolic ether extracts not only of tubercle bacilli, but also of saprophytic bacilli, and of other unrelated materials such as ox lungs (see Table V in Bloch, 1950).

It is disturbing, furthermore, that the toxicity for mice becomes apparent only after several successive injections of the material (which is a lipid and contains nitrogen despite earlier claims to the contrary). The technique of repeated injections in mice leaves open the possibility that the effects observed are not due to primary toxicity of the material, but instead to evocation of latent pulmonary viruses, or sensitization, or any number of other artifacts.

While there is as yet no valid information proving the existence of a toxin characteristic of virulent strains, a few well-documented observations might permit a fresh study of the problem of toxemia in tuberculosis. First is the fact that, in tissue cultures, virulent tubercle bacilli can reach large numbers intracellularly before killing the host cell (Maximov, 1928; Fell and Brieger, 1947; Suter, 1952*b*; Mackaness, 1954*a*, *b*; Mackaness, Smith and Wells, 1954). *In vitro*, at least, tubercle bacilli exhibit little primary cytotoxicity unless the cells are taken from a vaccinated (sensitized) animal.

It is possible to cause rapid death of mice by injecting into them large amounts of living virulent tubercle bacilli, 12 mg (wet weight) being required to cause acute death by intravenous injection (Youmans and Youmans, 1951). Interestingly enough, the acute lethal dose is the same irrespective of the virulence of the strain, and remains of the same order after the bacilli have been killed by phenol and washed with acetone and ether. In other words, the cells of H37Ra have a primary toxicity as high as those of BCG, R1Rv, or human and bovine virulent strains (unpublished observations).

It is well known that tuberculoproteins exert *in vitro* as well as *in vivo* a cytotoxic effect on cells of allergic animals; recent experiments indicate that mesodermic cells are more susceptible than epithelial cells in this respect (Rich and Lewis, 1932; Jacoby and Marks, 1953). The cytotoxic effect associated with allergy does not explain, however, the mechanism of caseous necrosis. Here again, there is no evidence that ability to cause caseation is related to virulence. Caseous necrosis ensues whenever tissues contain a sufficiently large number of bacilli, virulent or avirulent, living or dead. Although the precise chemical mechanism of caseation has not been worked out, enough observations are available to justify a working hypothesis (reviewed in Wells and Long, 1932; Weiss and Singer, 1953).

Tuberculoproteins cause, as we have seen, allergic necrosis of sensitized cells. In most other pathological conditions, necrosis is

followed by enzymatic lysis of the dead cells through the agency of their various autolytic enzymes. There is evidence, however, that some components of tubercle bacilli (virulent or avirulent) can inhibit proteolysis (Jobling and Petersen, 1914; Weiss and Singer, 1953). It has been suggested that the bacillary antiprotease is a lipid, but this has not been convincingly proved. In any case, it appears possible on the basis of present information that caseous material consists of the components of necrotic cells in various stages of autolysis, but characterized by the fact that certain proteins remain unhydrolyzed. It need not be emphasized that caseous necrosis constitutes one of the cardinal aspects of the pathology of tuberculosis, and that there is urgent need for a better understanding of the inhibitors of autolysis which are its primary cause, and of the phenomena which bring about the softening and liquefaction of caseous matter.

METABOLIC FACTORS AND THE FATE OF TUBERCLE BACILLI *IN VIVO*

Virulence and rate of intracellular multiplication. As we have seen, there is at the present time no evidence whatever that the difference in virulence between various strains of mammalian tubercle bacilli can be explained by their toxic properties. As far as is known, the cells of all strains, — virulent, attenuated or avirulent — possess the ability to cause acute or chronic toxic reactions, necrosis, and caseation. Differences in the power to cause disease probably derive, in part at least, from differences in ability to survive and multiply in the tissues.

There exist, in fact, several independent lines of experimental evidence showing that, for a given inoculum, the initial *rate* of multiplication of the bacilli in the tissues is one of the factors which determines the size of the microbial population eventually reached *in vivo*. Thus, from the very beginning of the infectious process, bovine bacilli multiply more rapidly than human bacilli in the rabbit, and still more rapidly than BCG (Lurie, 1928, 1953). Elaborate quantitative bacteriological studies have also estab-

lished a striking correlation between virulence (as measured by ability to produce lesions and cause death in guinea pigs and mice) and the initial rate and extent of multiplication of various strains of mammalian bacilli in mouse organs (Fig. 3; Pierce, Dubos, and Middlebrook, 1947; Pierce, Dubos, and Schaefer, 1953; Suter and Dubos, 1951).

Similar differences can be recognized by observing the fate of various strains of bacilli grown in rabbit monocytes in tissue cultures. On the one hand, the more virulent the culture, the shorter the lag period before it starts multiplying intracellularly. On the other hand, virulent bacilli appear to have a shorter generation time than attenuated bacilli within the monocytes (Mackaness, Smith, and Wells, 1954; Mackaness, 1954*a*, *b*). These findings suggest that the virulence of a given strain for a given host is determined in part, at least, by the rate at which it multiplies in the intracellular environment provided by the monocytes.

Studies *in vitro* have not yet gone far enough to provide a metabolic explanation of differences in behavior between strains. In culture media and under the proper conditions *in vitro*, all mammalian tubercle bacilli are capable of multiplying at the same rate. In other words, there seems to be no fundamental intrinsic difference in their replication mechanism. Indeed, when cultures three or more weeks old are transferred to a complete medium, they grow as readily as cultures in the logarithmic growth phase (Fenner and Leach, 1953). Yet, within monocytes, cultures of increasing age show a proportional increase in the lag period before they start to multiply, and old cells of attenuated strains (like BCG) may not grow at all, even though culture tests show them to be alive at the time of ingestion (Mackaness, Smith, and Wells, 1954). Clearly, then, monocytes do not provide optimum growth conditions for tubercle bacilli. Moreover, the growth rate of intracellular bacilli falls as the population density of the monocytes in tissue culture increases.

In vitro, the growth of tubercle bacilli is inhibited by certain organic acids (such as acetic, propionic, and lactic acids), particu-

larly where oxygen tension is reduced and that of CO_2 is increased. It is probable, therefore, that the metabolic activities of the tissue cells provide an unfavorable intracellular environment for the bacilli, especially under conditions of monocyte crowding. There are observations suggesting that the more attenuated the bacilli, the less resistant they are to the inimical conditions associated with anaerobic environment (Dubos, 1953*a*; Weiss and Dubos, unpublished observations). These differences may eventually find an explanation in the fact that acid-fast bacilli of various degrees of virulence seem to differ in metabolic activity when compared by the conventional manometric techniques.

Virulence and metabolic characteristics of the bacilli. In respiration tests using formate, acetate, propionate, butyrate, or lactate as substrate, virulent cultures are less stimulated than their avirulent or attenuated mutants, or than saprophytic acid-fast cultures. In a general way, there seems to be a negative correlation between vigor of oxidative attack and degree of virulence (Geronimus, 1949; Geronimus and Birkeland, 1951). Other studies have also shown that the cells of avirulent and attenuated strains have a higher rate of respiration than the virulent cells when tested in a variety of inorganic or complex organic media, either in air or at 1-percent oxygen tension (Heplar, Clifton, and Raffel, 1953).

There is evidence that one of the limiting factors in the metabolism of tubercle bacilli is a deficiency in their terminal oxidative system, that involving the tricarboxylic acid cycle (Geronimus, 1949). Yet metabolic intermediates do not accumulate in high concentrations in media in which the bacilli are growing, as would be expected if the citric acid cycle really failed to keep pace with the preliminary carbon-chain desmolysis. It has been suggested that intermediates do not accumulate extracellularly because the plasma membrane of acid-fast bacilli is poorly permeable and does not permit rapid diffusion from the intracellular into the extracellular environment. If this is true, an

inhibitory effect on tubercle bacilli might result either from the accumulation within the bacterial cells of toxic products of their intermediate metabolism, or from the saturation of certain of their carrier systems (Geronimus and Birkeland, 1951). It is hardly justifiable at the present time to develop further this working hypothesis with regard to the factors that might account for the quantitative differences recognized *in vivo* between virulent and attenuated bacilli.

Tissue metabolism and bacillary multiplication. Whatever their fundamental mechanism, the differences between virulent and attenuated strains of mammalian tubercle bacilli are only quantitative and not of kind. As already mentioned, R1Rv and BCG are able to multiply in the tissues of experimental animals and man; their multiplication is only less rapid and less extensive than that of the virulent cultures. In fact, it is possible to cause in mice (and probably in other animals) metabolic and nutritional disturbances that permit BCG infection to become progressive and fatal (Dubos and Pierce, unpublished observations). BCG will also produce fatal cavitary disease in silicotic guinea pigs (Vorwald *et al.*, 1950). It is of special interest in this respect that silicosis does not bring about a generalized increase in susceptibility to tuberculosis; only in the body areas affected by quartz dust do the attenuated bacilli find an environment suitable for continued growth. BCG infection evolves into disease in the silicotic individual, not because general resistance is depressed or because the bacilli are rendered more virulent, but because silicosis changes the physicochemical environment of the tissues in such a manner that the cells and body fluids offer no obstacle to bacillary growth, in contrast to what obtains in normal tissues.

It has long been held that monocytes are the main factors of resistance to tuberculous infection (Lurie, 1942, 1950; Suter, 1952*b*). But compelling objections, based on both old and very recent experimental findings, have recently been leveled against this theory (Mackaness, Smith, and Wells, 1954; Mackaness,

1954). While it is not possible at this time to formulate a final opinion concerning the existence and importance of cellular immunity in tuberculosis, it seems worth while to review briefly some of the facts which establish beyond doubt that bacillary multiplication *in vivo* is often inhibited by forces having their origin in the extracellular environment.

When virulent tubercle bacilli are injected into normal rabbits, the microbial population in the spleen at first increases, then becomes stabilized at a more or less constant, fairly low level. If, however, explants taken from the infected spleen tissue after the microbial population has become stabilized are cultivated *in vitro*, the bacilli start multiplying again and soon overgrow the tissue culture. In contrast, microscopic preparations reveal only a few scattered acid-fast bacilli in the spleen taken from animals sacrificed at the same time or later (Brieger, 1939). Clearly, then, inhibition of bacillary growth in the spleen *in vivo* depends upon some factor or combination of factors not possessed by the phagocytic cells in tissue cultures. It will be useful to consider another different type of experimental finding before discussing the nature of these factors.

Tubercle bacilli incorporated in an agar gel were placed in silk bags which had been impregnated with collodion in such a manner as to render them impervious to leucocytes, but still permeable to proteins and other soluble constituents of the plasma. The bags containing the bacilli were introduced into the peritoneal cavity of normal and tuberculous guinea pigs and rabbits. When they were removed two weeks later, histological examination revealed that the bacilli had multiplied extensively in the bags placed in the normal guinea pigs, whereas they had decreased in numbers in most of those in the tuberculous animals (Table 48). As phagocytic cells had not penetrated the bags, the difference in microbial population in the two groups of guinea pigs was obviously due to the antimicrobial activity of some humoral factor present in the tuberculous animals (Lurie, 1939). Although it is possible that this humoral factor was an antibody

TABLE 48

Fate of virulent bovine tubercle bacilli within collodion-impregnated silk bags placed in the peritoneal cavity of normal and tuberculous rabbits

Duration of stay of bags in peritoneal cavity (days)	Number of bacilli introduced in bags	Number of colonies recovered from bags in		pH of fluid from bags in	
		Normal rabbits	Tuberculous rabbits	Normal rabbits	Tuberculous rabbits
5	13,200	210,000	—	7.4	—
5	23,200	—	30,000	—	7.4
14	8,350	160,000	1,000	7.25	6.95
14	8,350	33,000	1,000	7.35	7.05
13	3,100	133,000	156,000	7.35	7.30
14	1,500	2,600	300	7.28	7.05
14	75,000	2,700,000	—	7.22	—
14	104,000	—	230,000	—	7.31

Condensed from Lurie, 1939.

of a nature as yet ill defined, other possibilities appear worth considering.

The bacilli and their metabolic products naturally called forth an inflammatory response around the silk-collodion bags. In the tuberculous animals in particular, the tuberculoproteins and tuberculopolysaccharides, diffusing through the bags, must have elicited immediate allergic reactions of both the tuberculin and the anaphylactic type, resulting in intense cellular exudate and in thrombosis of the vascular bed. This constellation of events must have rendered the local environment partially anaerobic and caused a predominantly glycolytic metabolism of the inflammatory cells — as evidenced by the low pH of the contents of the bags in the tuberculous animals (Table 48).

In no case was the pH in the bags sufficiently low to be responsible for inhibition of growth. But the increase in acidity probably went parallel with other biochemical changes. Since certain aspects of the inflammatory environment — increased

concentration of lactic acid and CO_2, decreased oxygen tension, presence of lysozyme, and so on — are inimical to tubercle bacilli as previously shown, it appears possible that the decrease in microbial population in the tuberculous guinea pigs was the result of physicochemical forces and not of a direct antibody action on the bacilli. It is of interest to note in this respect that of the bags in tuberculous animals, the only ones in which the numbers of bacilli remained high were those in which the pH was 7.25–7.3 (that is, as high as in the controls; see Table 48). Whereas the state of allergy (either of anaphylactic or of tuberculin type) caused an *early* reaction resulting in inhibition of growth in the tuberculous guinea pigs, the inflammatory response must have occurred later and been less intense in the normal animals, thus permitting bacillary multiplication to proceed uninhibited for a longer time.

It seems worth recalling here that crowding of monocytes in tissue cultures renders their intracellular environment ill suited to the multiplication of tubercle bacilli (Mackaness, 1954). It is not unlikely that crowding compels the phagocytic cells to a more anaerobic type of metabolism, which in turn interferes indirectly with bacillary growth.

The fate of bacilli in necrotic lesions. Necrosis and caseation bring into play still another set of factors which affect profoundly the fate of tubercle bacilli *in vivo*. When caseous material (taken at autopsy) is mixed with serum albumin in mineral medium and incubated *in vitro* with virulent bacilli under aerobic conditions, enormous bacillary growth ensues (unpublished observations; this, however, may not be true of all specimens). In contrast, there is no doubt that bacilli disappear progressively from closed caseous areas, even without chemotherapy (Rich, 1951; Canetti, 1946). This disappearance cannot be attributed to ordinary immunological mechanisms, since phagocytic cells do not function within caseous areas, and since bacillary proliferation begins again when plasma penetrates the lesion and liquefaction occurs. Caseous material constitutes a potentially ideal culture medium

for the bacilli; hence it must be concluded that the physicochemical environment of the closed lesion is unfavorable to them. In fact, it is certain that low oxygen tension, local acidity, presence of free fatty acids and of other antimicrobial agents liberated by autolytic processes during necrosis, readily account for failure of the bacilli to multiply within the lesion. Their ultimate disappearance, however, presents a somewhat more complex problem.

It is obvious that any organism prevented from synthesizing new protoplasm but placed under conditions where it continues to respire will progressively exhaust its metabolic reserves. This is the fate of tubercle bacilli in the closed necrotic lesions. Once their supply of reserve materials is depleted, energy is no longer available for the maintenance of cell structure, autolytic enzymes begin to function, and morphological and staining characteristics are lost. As can be readily demonstrated *in vitro*, the time required for the completion of this process varies enormously from one species of microorganism to another — a matter of hours for pneumococci, probably of months for tubercle bacilli. But before these irreversible reactions resulting in death and cellular disintegration take place, the organisms go through a phase during which they are still potentially capable of reproducing but poorly able to start multiplying *in vivo* or in ordinary culture media. This intermediary phase has been the source of many difficulties in the study of tuberculous lesions.

It has been repeatedly observed that necrotic material removed at autopsy or from surgical specimens may contain many acid-fast bacilli as revealed by microscopic examination, and yet fail to give growth on egg media or to infect guinea pigs (Canetti, 1946; d'Esopo *et al.*, 1953; Medlar, Bernstein, and Steward, 1952; Beck and Yegian, 1952). Although these bacilli are commonly regarded as dead, this is an assumption which cannot be proved by the bacteriological techniques presently available.

Like bacilli taken from old cultures, some of those present in closed necrotic lesions certainly exhibit a prolonged lag period (of weeks) before they start multiplying when transferred to a

new environment — *in vitro* or *in vivo*. It is well known, on the other hand, that the standard egg media become less favorable for bacterial growth and even toxic on aging. Therefore, these media do not lend themselves well to the cultivation of bacilli whose growth is much delayed by reason of metabolic deficiencies. Nor are the usual infection tests in guinea pigs better suited to determine the viability of old bacilli. Even after having been kept for only a very few weeks in liquid media, bacilli display a detectable lag period before they begin to multiply within phagocytic cells in tissue cultures (Mackaness, 1954*a*, *b*; Mackaness, Smith, and Wells, 1954). As phagocytosis is extremely rapid *in vivo*, the first phase of tuberculous infection is intracellular. It can well be assumed, therefore, that living, but old, bacilli often fail to establish infection because the intracellular environment is unfavorable to them.

Still another fact, which cannot be developed here, adds further difficulty to the initiation of growth with bacilli taken from old lesions. It will be recalled that tissue necrosis brings about the release of a variety of bacteriostatic substances. Many of these substances probably are adsorbed on the bacilli and are introduced with the inoculum into the culture medium or at the site of experimental infection, thus retarding, or at times preventing altogether, bacillary multiplication *in vitro* or *in vivo*.

But failure to grow in a certain environment does not necessarily mean inability to grow in another, as repeatedly demonstrated with other microbial species (reviewed by Dubos, 1945, p. 286). For example, bacteria paralyzed by treatment with mercuric poisons will grow if sulfhydryl compounds are added to the culture medium. We have also reported in an earlier chapter experiments showing that suspensions of colon bacilli rendered unable to grow in ordinary nutrient broth by treatment with heat, chlorine, peroxide, ethyl alcohol, or detergents yield large numbers of colonies when incubated in the presence of metabolites of the Krebs cycle (Heinmets, Taylor, and Lehman, 1954; see Table 10). Similarly, it is almost certain that tubercle bacilli

which remain dormant under certain conditions may slowly start multiplying under other conditions. Metabolic disorders may, for example, lead to the temporary accumulation *in vivo* of substances which favor initiation of growth; polycarboxylic or keto acids are but one group of such substances which we have already considered (see pages 30 and 39, Tables 9, 15, and 17; see also page 31 and Table 11). On the other hand, immunological reactions, as well as a variety of nonspecific events, are known to bring about the activation of serum and tissue proteases (see pages 91 and 119) and perhaps of other enzymes. This may result in breakdown of certain parts of organized lesions, for example, of collagenous structures, and change thereby the physicochemical characteristics of the local environment in such a manner as to favor multiplication of the organisms present in the tissues in a latent state.

Tissue factors and chemotherapeutic failures. Local conditions are also of paramount importance in determining the efficacy of antimicrobial drugs and the emergence of drug-resistant forms of the bacteria.

It is a puzzling fact that drugs which are powerfully bacteriostatic for tubercle bacilli *in vitro*, and therapeutically effective *in vivo* against other microbial agents, are of little if any value in the treatment of human tuberculosis; the sulfonamides and chloramphenicol are examples of this discrepancy. On the other hand, while it is relatively easy to arrest the progress of tuberculous infection with streptomycin, para-amino salycilic acid, or isoniazid, even prolonged treatment with these drugs often fails to sterilize necrotic lesions. Obviously, there are many factors of the *in vivo* environment which affect profoundly the antimicrobial activity of drugs, and we can mention only a few of them.

Drugs which do not penetrate living phagocytic cells or are inactive in the intracellular environment can control only the extracellular phase of tuberculous infection; this is, in part at least, the case with streptomycin (Mackaness, 1952; Suter,

1952*a*). The pH of the environment in which drugs and bacilli come into contact is of extreme importance; thus, streptomycin and sulfonamides exhibit very little activity even *in vitro* at the slightly acidic reactions likely to prevail in many types of lesions. There exist in tissues a number of substances which inhibit the antimicrobial activity of drugs, para-amino benzoic acid, nucleic acids, and keto acids and pyridoxal being, for example, inhibitors of PAS, streptomycin, and isoniazid, respectively. Necrosis, in particular, results in the release locally of a large variety of intracellular components and breakdown products of tissues which almost certainly contribute to the failure of drugs to act in established lesions. Caseation exaggerates this effect by allowing the persistence of partially hydrolyzed tissue components, some of which probably act as metabolic antagonists. Moreover, it is known that many drugs are most effective when acting on microorganisms in a proliferating state; isoniazid, for example, has virtually no bactericidal effect on resting bacilli (Schaefer, 1954) and therefore must be ineffective in closed lesions where multiplication has stopped.

The many factors which interfere with the effectiveness of drugs *in vivo* are of obvious importance in explaining the failures of chemotherapy. They account, moreover, for the difficulty in preventing the emergence of drug-resistant forms of the bacteria during treatment. According to genetic theory, the likelihood of an organism developing simultaneously resistance to two different drugs is vanishingly small. The validity of the theory is confirmed by the fact that cultures of tubercle bacilli do not become resistant to either streptomycin, or PAS, or isoniazid when incubated in media containing any combination of these drugs. In principle, therefore, it appears easy to prevent the emergence of drug resistance during therapy by treating simultaneously with two or more drugs. Unfortunately, the results in practice are far less satisfactory than would be expected from theory. The simultaneous use of several drugs in combination delays somewhat the emergence of resistance, but it hardly ever

prevents it. The reason for this discrepancy between theory and practice is to be found in the fact that theoretical tests *in vitro* are carried out under conditions which allow drugs and microorganisms to come into contact in a homogeneous environment, whereas the infectious process *in vivo* takes place in many different environments, isolated one from the other, each antagonizing to a different degree the antimicrobial activity of the various drugs. For example, even though both streptomycin and isoniazid be simultaneously present in a given area in adequate concentrations, many circumstances arise in which only one of the two drugs is active. Keto acids or other metabolic antagonists may render isoniazid inactive while allowing streptomycin to function. In contrast, free nucleic acids or a low pH in the tissue may selectively inhibit streptomycin and not isoniazid. As streptomycin is inactive within phagocytic cells, the bacilli present intracellularly are influenced only by isoniazid, even though streptomycin be administered in high doses and is effective against the extracellular phase of the infection.

Similar examples could be cited to account for the fact that combined treatment with two or even more drugs often fails to prevent the emergence of drug-resistant forms. But it would not be profitable to elaborate further on this subject, the circumstances which bring about selective antagonism against one or another drug being so varied as to defy analysis at the present time. It will be sufficient to point out once more that the problems of pathogenesis and therapy of infection cannot be analyzed exclusively in terms of general immunological or physiological characteristics of the host and the parasite, because each local environment presents physicochemical peculiarities of its own which affect selectively the effectiveness of immune mechanisms and antimicrobial agents. Although it is in the case of tuberculosis that local tissue factors appear of greatest importance in determining the outcome of the infectious process, these factors also play a large part in all other microbial diseases, particularly in infections exhibiting an intracellular phase and extensive necrosis (McDermott, 1949).

CHAPTER 5

Immunity and inflammation

The antimicrobial agencies normally present in the tissues and body fluids come into play shortly after the infective agent reaches the host, and usually succeed in interrupting its proliferation. If the microbial population continues to increase, however, there occurs within a few days the complex of histological and biochemical reactions collectively known under the name of immunological response. Needless to say, this response presents characters peculiar to each type of infection and to each host, and cannot be discussed properly in terms applicable to all situations. Nevertheless, a few general remarks may help in defining more precisely the place of the phenomena of immunity in the framework of the infectious process.

Some aspects of specific acquired immunity are now fairly well understood. The protective role of antitoxins in primarily toxic infections, or of specific opsonins against microorganisms which are killed within phagocytic cells, has an appealing simplicity which has made these aspects of immunology popular among investigators and textbook writers.

Antibodies directed against bacterial capsules, or against exotoxins, represent, however, only a very small part of immune mechanisms. They provide no explanation in particular for immunity against infections caused by intracellular parasites — and these constitute the largest group of microbial diseases. There is no evidence to date concerning the mode of action of antibodies against viruses or rickettsia. Indeed, it is rather puzzling that in some cases — herpes simplex, for example — the virus can per-

sist for years and cause lesions at fairly frequent intervals, in persons possessing high titers of neutralizing antibody, whereas in other cases — as in psittacosis — a high level of immunity can exist in the absence of detectable protective antibody.

It is always possible, of course, to resort for an explanation to that catchall of immunological doctrine, cellular immunity. The theory of cellular immunity has taken many different forms in the course of time, the last being based on analysis of the phenomenon of lysogeny (Lwoff, 1953). There is evidence that a certain developmental stage of bacteriophage, which has been called prophage, can occupy a specific site in susceptible cells, and thus prevent infection by the virulent form of the bacterial virus. This protective mechanism is akin to the process of interference in viral diseases of animals, but it is possible that the analogy is purely formal, since immunity in higher organisms calls into play many reactions which do not occur in unicellular organisms.

It is always stimulating to discuss the possible role of cellular immunity in animal infections. But if such a property exists — independently of humoral factors — it is extremely elusive and behaves as a will-o'-the-wisp fading away in the light of searching experimentation! Cellular immunity was regarded as the only mechanism of resistance in malaria until really effective techniques became available for the production and measurement of antiplasmodial antibodies (Coggeshall, 1943). The theory of cellular immunity still holds sway in the field of tuberculosis, but the latest and apparently most objective methods of observation have thrown much doubt on the significance of earlier findings. When the fate of tubercle bacilli is followed by direct examination with contrast microscopy, it is found that, in tissue cultures, bacillary multiplication is just as rapid in the cells of immune as in the cells of normal rabbits (Mackannes, 1954*a*, *b*).

The failure of present-day doctrines to explain any of the aspects of chronic infections is so obvious as to require no comment. Indeed, it is plain that the microbial diseases for which

there is no explanation whatever of immunity mechanisms far outnumber those where susceptibility and resistance can be explained in terms of recognized immunological reactions — cellular or humoral. For this reason, speculative thoughts may be permitted in this field, so barren of facts and even of theories.

It had been suggested in the early days of bacteriology that acquired resistance could be explained by assuming that the first bout of infection depleted the host of some factor essential to the growth of the parasite concerned. It is unlikely that this "exhaustion" theory ever applies, even in cases where the infectious agent depends for its multiplication on some substance which is in extremely short supply *in vivo*. During recent decades, the nutritional explanation of acquired resistance has taken a different form in the theory of antiblastic immunity. According to this theory, immunity operates by starving the infectious agent through some interference with its nutritional processes. In a few infections caused by helminths and protozoa a specific antibody — anablastin — coats the surface of the parasite and may thus interfere with the penetration of food into it (Taliaferro, 1932, 1948). Although no evidence exists that antiblastic immunity operates in other microbial diseases, some related facts suggest that the possibility may be worth considering.

All known enzymes are proteins and most of them probably behave as antigens. It has been shown, furthermore, that injection of certain enzymes into experimental animals elicits the production of antibodies which not only can precipitate the enzyme protein, but also can inhibit its enzymatic action. Anticatalase, antilecithinase (anti Welch alpha toxin) are examples of such anti-enzymes. As some of the enzymes concerned in intermediate metabolism, or in the hydrolysis of nondiffusible substrates, are located at or near the plasma membrane, it seems possible that antibodies against them might be produced during the multiplication of the microbial agent *in vivo*. Thus, immunity processes might interfere with the metabolism of microorganisms by inactivating certain of their surface enzymes, and causing a

kind of metabolic disturbance very different in mechanism from the common bacteriostatic and bactericidal effects.

The observations presented in the preceding chapters make it likely, on the other hand, that acquired resistance can also operate not through a direct action on the infective agent, but by creating around it an environment unfavorable to its survival, or to its multiplication, or to the expression of its toxicogenic potentialities. One of the most constant and general effects of the immunological response is to accelerate, and intensify, the inflammatory processes around the infective agent and its products. In very general terms, and at the cost of some repetition, it seems worth reviewing in this light the effects of inflammation on the course and outcome of infectious diseases.

We cannot consider here the multiple agencies which elicit the inflammatory response during infection: the release by microorganisms of substances chemotactic for leucocytes, the antigen-antibody as well as allergic reactions with their attendant local production of pharmacologically active substances; the various influences controlling diapadesis, and so on. We shall instead limit our discussion to the bearing of the inflammatory response on host-parasite relationships.

Some of the effects of inflammation appear at first sight essentially physical — fibrin deposition, for example. On the one hand, the fibrin network provides interlacing pathways throughout the tissue spaces distended with fluid, thus permitting the phagocytes to move readily and to accumulate at the site of infection. On the other hand, the fibrin clots tend to block the various channels and to wall off the infected area, thus retarding the spread of microorganisms.

There is a tendency to give to the expression "walling off" too literal a meaning. In reality, experiments *in vitro* have revealed that fibrin films are remarkably selective in their permeability (Table 49). Needless to say, permeability is affected by the conditions under which the clot was formed and by the pressure used for the filtration tests. Nevertheless, certain generaliza-

TABLE 49

Permeability of fibrin membranes

Material tested	Passage at 600 mm Hg pressure
Trypan blue	No
Vital new red	Yes
Staphylococcus toxin	No
Diphtheria toxin	Yes, slowly
Serum albumin	Yes
Serum globulin	No
Diphtheria antitoxin	Very slowly
Polymorphonuclear leukocytes	Yes
Bacteria	No

Condensed from Hughes, 1948.

tions appear justified. When serum is filtered under pressure or diffuses across a fibrin membrane, albumin passes through and globulin is concentrated behind the membrane. Thus, the filtrate has a composition similar to that of inflammatory exudates, with a lower total protein content and reversal of the albumin-globulin ratio. Of particular interest is the fact that antibodies, being globulins, do not go through the membranes. Some toxins (that of diphtheria, for example) diffuse slowly, while most bacterial products do not at all. Bacteria themselves are held back by the fibrin membrane but leucocytes pass readily through them (Hughes, 1948).

In view of these facts, it is obvious that the presence of fibrin coagulum is of enormous importance for determining the chemical and biological characteristics within the lesion. Fibrin acts as a barrier between certain microbial and tissue components and thus prevents them from coming into contact; it interferes in many ways with a variety of immune mechanisms. Nothing is known of the selective permeability of other types of pathological structures which are also deposited as a result of inflammation, but one can well assume that they too play a part in giving to localized

foci a physicochemical character very different from that of the body as a whole. On the other hand, fibrin clots, as well as other pathological structures, can be attacked by enzymes of the infectious agents, and even more readily, perhaps, by enzymes of the tissues themselves. Because of this, the role of protease inhibitors and activators is certainly of enormous importance in controlling the fate of lesions (see page 91). Here lies one of the virgin fields of experimental pathology, conceptually and technically difficult of approach, but of vast potentialities for the understanding of pathogenesis.

We have already discussed how phagocytosis can affect the infectious agents, cause their death, favor their proliferation, or change their properties. We shall review briefly now some of the effects that inflammatory cells have on the physicochemical characteristics of the lesion. Even in the presence of oxygen, phagocytes have a predominantly glycolytic mechanism which is exaggerated by the oxygen deficiency resulting from the interference with circulation caused by fibrin clots and vascular damage. The amount of carbohydrate in the inflammatory area falls to a very low level, lactic acid and CO_2 accumulate, K^+ ions are released, the osmotic pressure increases — all these changes affect not only the infective agents, but also the activities of host cells.

We have seen that certain organic acids, lactic acid in particular, can prevent the growth and even cause the death of many types of microorganisms, especially when the conditions are partially or totally anaerobic. Eventually, the leucocytes as well as other cells also are injured by components or products of the infective agent, and by the physicochemical factors of the inflammatory area. Necrosis leads to the release of a large variety of cellular constituents or breakdown products, many of which possess marked antimicrobial properties (complement, lysozyme, basic peptides, heme compounds, long-chain fatty acids, and so on).

Granted that this general schema fits no particular case, it

appears that the inflammatory process tends to isolate the site of infection, to bring to it a variety of wandering cells, to change the activity of fixed tissue cells, and in addition to concentrate locally many types of anti-infectious agents of cellular origin. As a result of this constellation of effects, infective agents rarely increase in number in the inflammatory necrotic areas, even when they are potentially capable of intracellular life. Extracellular death and dissolution of virulent pneumococci is observed early in the central parts of the pneumonic lesion while the lobar pneumonia is spreading rapidly; staphylococci become scarcer in old abcesses while new abcesses may be forming in other parts of the body; the bubos in bubonic plague or the syphilitic gummas contain remarkably few bacilli or spirochetes, and a similar state of affairs can be observed in the comparable lesions of tuberculosis.

Thus, the spread of infective agents from one part of the body to another may be restrained, not only through the ordinary processes of immunological reactions (humoral or cellular), but also through the existence at the focus of infection of a physico-chemical environment which interferes with their survival and multiplication. Hormonal control, the physiological state, anatomical situations, allergic processes, constitute circumstances which give peculiarities to each particular lesion, and hence to the environment in which is determined the fate of the infective agent. In this sense, it is possible to speak of a local immunity different from the general immunological state of the body, and yet influenced by it. This accounts for the commonly observed fact that the infective agent can thrive and produce progressive disease in one part of an organ, while being held in check in another area of the body, even indeed within the same organ.

So many, and often so effective, are the antimicrobial agencies mobilized at the infected site before and after the development of immunity that one might regard the inflammatory response as a homeostatic reaction, a manifestation of the wisdom of the body designed to maintain the *status quo* against noxious influences from the outside world. Needless to say, this teleological view

represents only one aspect of the influence of inflammation on host-parasite relationships. There are many cases where inflammatory processes, far from contributing to the destruction of the pathogenic agent, provide for it an environment in which it can multiply or find a shelter against antimicrobial substances — either released by the host tissues or introduced during therapy. We have already mentioned as illustration of this statement the fact that the intracellular environment provided by phagocytosis favors multiplication of many microorganisms and protects them against antibodies and drugs — also that some of the autolytic products released by necrosis often constitute a good culture medium for microorganisms and can, furthermore, antagonize the antimicrobial activity of many drugs. It is also well known that inflammation can bring about the release of products toxic to the host, or the destruction of some of its tissues. In the eye, for example, its price may be the permanent loss of function. Tuberculosis provides many examples of the wisdom of the body gone astray.

Even though inflammation frequently has unfavorable effects on the individual, it helps in overcoming infection probably more often than it enhances disease. If its results could be expressed statistically, inflammation would be found in all likelihood to have survival value for the species. In infectious diseases, just as in other physiological and pathological processes, the wisdom of the body is not concerned with the well-being of one individual at a given time, but expresses itself rather in reactions which permit the collectivity — the species or the group — to function, survive, and reproduce itself in a given environment. The importance of inflammation as a protective mechanism against microbial diseases can be measured only on the evolutionary scale.

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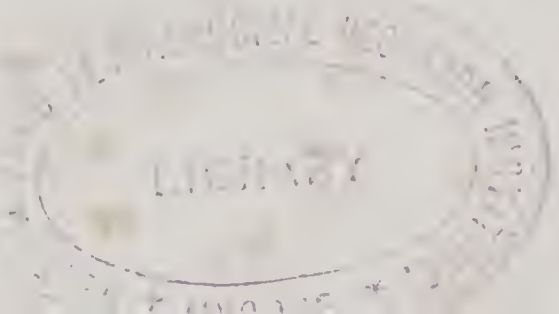

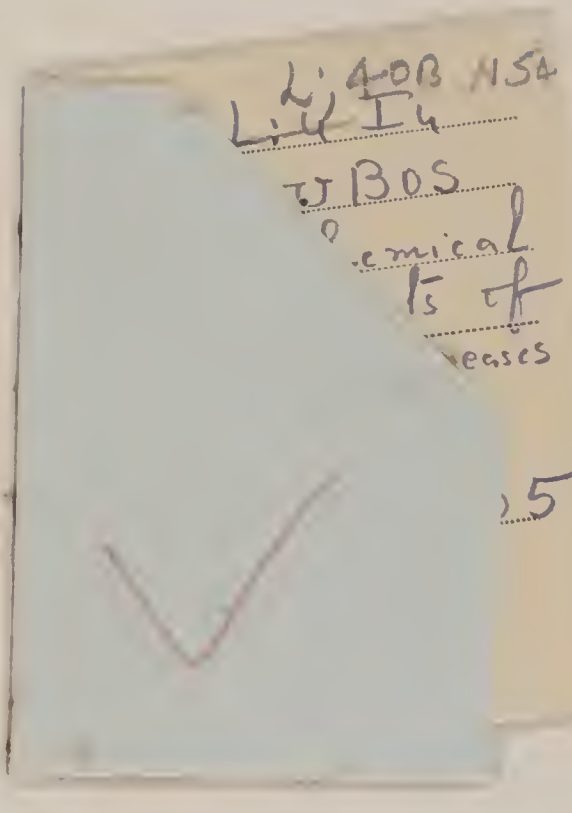
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